TRANSCRIPTOMIC REGULATION OF EGG FORMATION IN THE OVIDUCT OF HENS

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ABSTRACT

Introduction: The oviduct of a hen provides a conducive environment for egg formation. Each part of the oviduct has a unique function: infundibulum (receives egg), magnum (deposits albumen), isthmus (synthesizes eggshell membranes), uterus (forms mineralized eggshell), and vagina (helps in oviposition). Any functional anomaly of the oviduct will lead to small-sized, misshapened, soft-shelled or cracked eggs, and taken together; these defects account for lost revenue in approximately 10% of the total number of eggs produced. At present, the cellular processes and biological pathways involved in the albumen synthesis, and eggshell formation are not clearly understood.

Objective: The purposes of this study were to: (1) identify the novel differentially expressed genes (DEGs) and important biological pathways in the oviduct (laying vs. non-laying), and (2) validate the identified novel genes in the laying (3 h and 15-20 h post-ovulation; p.o.), non-laying (no growing ovarian follicles), and molting (reproductive rest) hens.

Methods: Hy-line white hens, including laying (n=12) of 35 weeks, non-laying (n=6) of 35-60 weeks, and molting (n=6) hens of 60 weeks were used for this study. Magnum and uterine tissues were collected from laying hens at 3 h p.o. (egg present in the magnum), and 15-20 h p.o. (egg present in the uterus), molting, and non-laying hens for total RNA isolation. Total RNA (n=3/group) from the magnum of laying (3 h p.o.) versus non-laying, and total RNA (n=3/group) from uteri of laying (15-20 h p.o.) versus non-laying hens were subjected to next-generation sequencing (NGS). Raw reads of the sequences were filtered and trimmed (to remove low-quality reads), then aligned with the chicken genome (Galgal 5.0) and analyzed for differential gene expression. The mRNA expression of selected candidate genes in the magnum and uterus of laying, non-laying, and molting hens were also validated using real-time quantitative PCR (qPCR).

Results: A total of 152 genes in the magnum, and 229 genes in the uterus of laying hens were up-regulated (FDR_BH <0.05) with the presence of egg in the oviduct. In the magnum, the glycine, serine and threonine metabolism was the most-enriched pathway; whereas, in the uterus, calcium signaling was amongst the most prevalent pathways. Results of qPCR confirmed the higher (P <0.05) mRNA expression of *AVD* and *AvBD11* (antimicrobials); *CAPN2*, *TMPRSS9*, *MMP1*, and *MMP9* (ECM remodeling and angiogenesis); *REN* and *RLN3* (albumen secretion and oviposition); and *CGN* (vascular permeability) in the magnum of layers compared to both molters and non-layers. In the uterus, mRNA expression of *MEPE* (regulator of mineralization), *OTOP2* (modulator of cellular calcium influx), *CALCB* (intracellular release of Ca-ions), *STC2* (increases alkaline phosphatase activity), and *ATP2C2* (cellular import of Ca-ions) were significantly higher (P <0.05) in laying hens, when compared to either molting or non-laying hens.

Conclusions: This study identified several novel genes and biological pathways involved in the albumen synthesis and eggshell formation. These molecules can potentially be used as markers to improve the egg-qualities through nutritional modulation, and genetic improvement.

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LIST OF ABBREVIATIONS

ACE	Angiotensin-converting enzyme
ACP	Acid phosphatase
ANOVA	Analysis of variance
ASNS	Asparagine synthetase
ATG	Autophagy-related
ATP	ATPase
AvBD	Avian beta-defensin
AVD	Avidin
CALB	Calbindin
CALCB	Calcitonin related polypeptide beta
CAMK1D	Calcium/calmodulin-dependent protein kinase
CAPN	Calpain
CGN	Cingulin
COL	Collagen
CREMP	Cysteine rich
CYP	Cytochrome P 450
DE	Differentially expressed
DEG	Differentially expressed gene
DNA	Deoxyribo nucleic acid
ECM	Extracellular matrix
ESM	Eggshell membranes
EtBr	Ethidium bromide
FBN	Fibrillin

FSH	Follicle stimulating hormone
GAPDH	Glyceraldehyde 3-phosphate dehydrogenase
GEO	Gene expression omnibus
GNRHR	Gonadotropin-releasing hormone receptor
GO	Gene ontology
GPX	Glutathione peroxidase
IPA	Ingenuity pathway analysis
IP3	Inositol triphosphate
KEGG	Kyoto encyclopedia of genes and genomes
LH	Luteinizing hormone
LOX	Lysl oxidase
LYZ	Lysozyme
MELTF	Melanotransferrin
MEPE	Matrix extracellular phosphoglycoprotein
MMP	Matrix metalloprotease
NGS	Next Generation Sequencing
OC	Ovocleidin
OCX	Ovocalyxin
OSTN	Osteocrin
OTOP	Otopetrin
OVL	Ovalbumin
OVST	Ovostatin
p.o.	post ovulation
PCR	Polymerase chain reaction

PENK	Proenkephalin
PHGDH	Phosphoglycerate dehydrogenase
PROC	Protein C
PSAT	Phosphoserine aminotransferase
PSPH	Phosphoserine phosphatase
qPCR	Quantitative polymerase chain reaction
QSOX	Quiescin Q6 sulphydryl oxidase
RCAN	Regulator of calcineurin
REN	Renin
RLN	Relaxin
RNA	Ribonucleic acid
RNA-Seq	RNA-Sequencing
ROS	Reactive oxygen species
SAS	Statistical analysis system
SLC	Solute carrier
SPP	Secreted phosphoprotein
STC	Stanniocalcin
TBE	TRIS borate EDTA
TBP	TATA box biding protein
TF	Ovotransferrin
TMPRSS	Transmembrane serine protease
TXN	Thioredoxin
UVJ	Uterovaginal junction

CHAPTER 1: LITERATURE REVIEW

1.1 The hen's oviduct

The avian oviduct is a complex tissue, with dynamic cellular activity, and represents the site where the ovulated yolk from the ovary is developed into the egg. In contrast to mammals, poultry, specifically hens, have a single set of functional reproductive organs, the left ovary, and the oviduct. When a chick hatches, the development of the right ovary and oviduct cease and gradually regress in the first postnatal week. The oviduct itself is a long tubular structure consisting of five functionally distinct parts namely the: infundibulum (site of fertilization), magnum (production of the components of egg white), isthmus (formation of the soft shell-membrane), shell gland or uterus (formation of calcified eggshell) and vagina (oviposition or egg laying). Following ovulation, the ovum passes through the entire length of the oviduct, where the constituents of the egg are secreted and deposited from respective parts of the oviduct.

The deposition of the egg constituents around the yolk is both time- and tissue-specific. Each part of the oviduct secretes a unique component to the egg and the egg spends a fixed duration in each segment of the oviduct. The ovulated yolk remains in the infundibulum for a very brief period, about 15-30 minutes. Then in the magnum, the egg-white protein is accumulated over the yolk for 2-3 h when the egg traverses the magnum. Once the egg reaches the isthmus, it spends 1-2 h during which the outer- and inner- eggshell membranes envelop the albumen. Eventually, the egg descends into the uterus, where it will remain for nearly 18-20 h. During this period, the calcite crystals are mineralized with the organic matrix over the periphery of the eggshell membrane. This outermost calcified covering of the egg is the eggshell, which provides physical and biological protection to the contents of the egg. In this manner, a complete egg is formed in the oviduct of a hen in about 22-26 h.

Two recent studies reported numerous genes associated with albumen deposition, eggshell membrane formation and shell calcification (Du et al., 2015; Willems et al., 2014). As shown in Table 1, gene expression and protein secretion in the oviduct varies in accordance with functional roles specific to different regions of the oviduct. During the formation of the egg, oviductal tissues undergo extensive proliferation of luminal and glandular epithelial cells, extracellular matrix remodeling, and neovascularization (Jeong et al., 2012). Therefore, it is important to understand the oviductal genes/proteins involved in the egg formation.

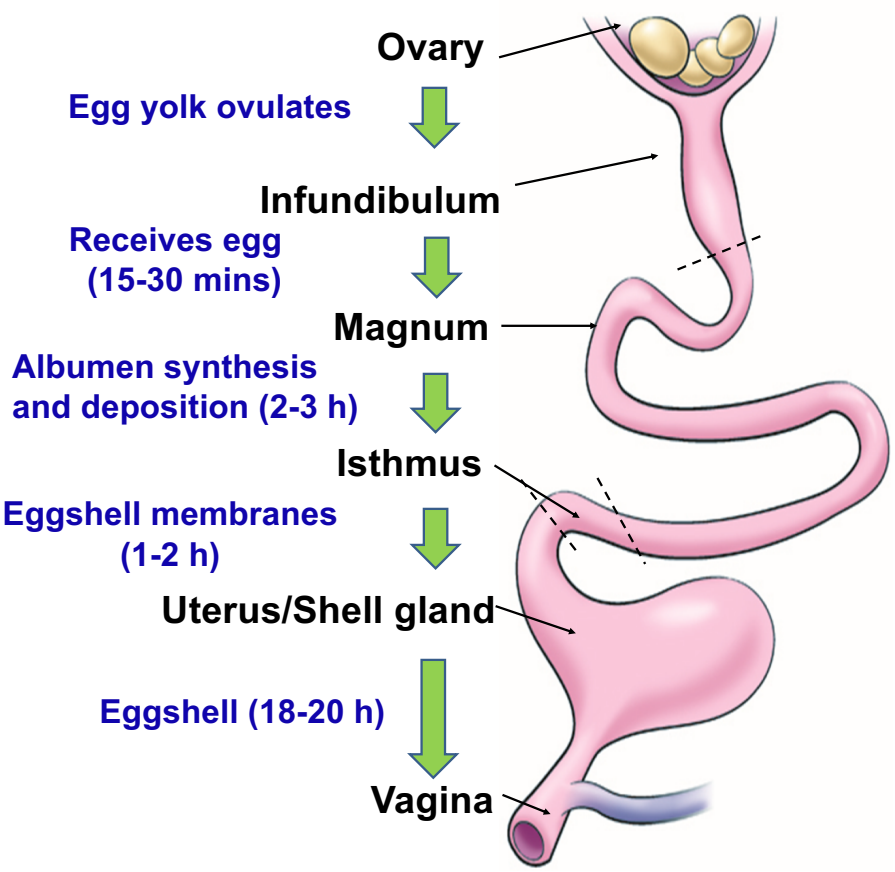

Figure 1 Schematic diagram of the poultry oviduct. (adapted from Hobby Farms magazine)

1.2 Functional role of the oviduct

1.2.1 Infundibulum

The infundibulum is a muscular funnel-shaped structure marking the beginning of the

oviduct. It is in close proximity to the growing follicles and surrounds them so that the ovulated follicle is trapped and directed to descending parts of the oviduct. The infundibulum is the site of any possible fertilization of the egg. The infundibulum has no secretory functions, and the egg remains here for a period of 15 to 30 minutes. However, if the infundibulum malfunctions, the follicle may ovulate outside of oviduct into the internal peritoneal cavity. A study conducted on Brown- Leghorn pullets revealed that when this 'internal laying' was evident, there was a loss of 12% of potential eggs (Wood-Gush and Gilbert, 1970).

1.2.2 Magnum

The magnum is the longest part of the oviduct and contributes the egg white protein to the developing egg during its 3 h of passage through this segment. Synthesis of albumen occurs continuously in the tubular gland cells of magnum, however, the secretion rate is heightened when the egg is present in this region (Muramatsu et al., 1991). Albumen provides an antimicrobial function prior to egg incubation, and its proteins are the primary source of nutrition for the growing embryo during incubation (Willems et al., 2014). Among the several egg white proteins, including ovalbumin, ovomucin, and conalbumin, ovalbumin constitutes around 54% of the total egg white proteins. Genes/proteins expressed in the magnum such as avidin, avian beta-defensins, and lysozyme (Table 1) are incorporated in the egg and protect the embryo during storage. The albumen alone constitutes more than 50% of the total egg-weight. The amount of the albumen deposited also determines the size of the egg. Therefore, it is important to understand the transcriptomic regulation of albumen synthesis and secretion for improving egg quality and food safety characteristics.

1.2.3 Isthmus

The isthmus bridges the magnum and the shell gland in the chicken oviduct. When the inchoate egg passes through the isthmus, the eggshell membrane (ESM) components are secreted

and assembled around the periphery of the egg white. Besides covering the soft inner structures, the ESM provides a structural foundation for eggshell calcification. The ESM is a transparent film primarily composed of fibrous proteins such as collagen. The expression of *COL10A1*, *FBN1*, *CREMP*, *LOX*, *TXN*, and *QSOX1* in the isthmus are involved in the formation of ESM (Corson et al., 1993; Kodali et al., 2011; Du et al., 2015) as listed in Table 1. However, the mechanism of formation of ESM in the isthmus is not completely understood.

Table 1 Summary of the expression, localization, and functions of the genes in the laying hen oviduct.

Gene	**Tissue**	**Function**	**References**
OVAL, OVALX, OVALY	Magnum	Deposition of egg white	Hrabia *et al*., 2014; Joensuu *et al*., 1991; Jung *et al*., 2011; Socha *et al*., 2017; Zhao *et al*., 2016
AVD	Magnum	Biotin transfer to egg	Jung *et al*., 2011
LOC	Magnum	Trypsin inhibitor, Antiviral, Antibacterial, Antitumor	Kobayashi *et al*., 1994; Jung *et al*., 2011; Tsuge *et al*., 1997
SPIK5	Magnum	Serine protease inhibitor, Antimicrobial	Bourin *et al*., 2011; Feeney, 1963; Kim and Choi, 2014
OVST	Magnum	Proteinase inhibitor	Kitamoto *et al*., 1982; Lim *et al*., 2011
LYZ	Magnum	Antimicrobial	Jung *et al*., 2011
TF	Magnum and Isthmus	Antimicrobial, Antifungal, Anticancer, immunomodulator	Kim and Choi, 2014
COL10A1	Isthmus	Formation of fibrous eggshell membranes	Du *et al*., 2015; Rath *et al*., 2017; Wang *et al*., 2002
FBN1	Isthmus	Provides elasticity to eggshell membranes; calcium-binding	Corson *et al*., 1993; Du *et al*., 2015
CREMP	Isthmus	Antimicrobial; provide thio-substrate for enzymatic reactions	Du *et al*., 2015; Kodali *et al*., 2011
LOX	Isthmus	Cross-linkage between eggshell membrane proteins	Du *et al*., 2015; Sun *et al*., 2013
TXN	Isthmus	Reduction of protein thiol group	Du *et al*., 2015; Housley *et al*., 1984
QSOX1	Magnum, Isthmus, Uterus	Catalyzes the formation of ESM proteins cross-linkage	Du *et al*., 2015; Mann, 2008; Miksik *et al*., 2010
CALB1	Uterus	Calcium ion binding	Arazi *et al*., 2009; Jeong *et al*., 2012; Jonchere *et al*., 2012; Nys *et al*., 1992; Yosefi *et al*., 2003

CYP26A1	Uterus	Heme binding; Iron ion binding; retinoic acid 4-hydroxylase activity	Brionne *et al*. 2014; Jeong *et al*. 2012
PENK	Uterus	Opiod peptide activity	Brionne *et al*., 2014; Jeong *et al*., 2012
SPP1	Uterus	Extracellular matrix binding	Arazi *et al*., 2009; Jeong *et al*., 2012; Lavelin *et al*., 1998; Lim *et al*., 2012b; Pines *et al*., 1995
RCAN1	Uterus	Serine threonine protein phosphatase activity	Jeong *et al*., 2012
OCX-32, -36, and -21	Uterus	Biomineral tissue development, antimicrobial activity, pH buffering for rapid calcification	Brionne *et al*., 2014; Gautron *et al*., 2001; Hernandez-Hernandez *et al*., 2008; Hrabia *et al*., 2014; Jonchere *et al*., 2010
OC-17, and -116	Uterus	Biomineralization and antimicrobial activity	Hincke *et al*., 1996, Jonchere *et al*., 2010; Reyes-Grajeda *et al*., 2004
ACP1	Magnum and Uterus	Acid phosphate activity; Protein tyrosine phosphatase activity	Ek-Rylander *et al*., 1994; Jeong *et al*., 2012
AvBD-1 to AvBD-14	Entire oviduct	Antimicrobial activity	Mageed *et al*., 2009; Yoshimura *et al*., 2006
MMP-2, -7, and -9	Entire oviduct	ECM remodeling	Hrabia *et al*., 2013; Lesniak-Walentyn and Hrabia, 2016a; Mishra *et al*., 2012; Vu and Werb, 2000
SLC (1A4, 13A2, 35B4, 4A5, 7A3, 41A2, 42A3, 25A30, 12A8, 16A2, 4A9)	Magnum and Uterus	Transport of glucose, amino acids and electrolytes	Atikuzzaman *et al*., 2015, 2017; Brionne *et al*., 2014; Lim *et al*., 2012a

1.2.4 Uterus (shell gland)

The uterus in hens is commonly known as the shell gland which consists of numerous glands. The glandular secretions function to provide the outer calcified, protective layer for the chicken embryo. The egg remains in the uterus for the longest period during formation, for a duration of 20-22 h, during which eggshell calcification occurs in three stages; initiation, growth, and arrest. Calcification initiates from nucleation sites on the ESM. During the initiation stage, the rate of calcification is slow; however, it gets accentuated over 15 h and ebbs down to a nadir in the last two hours before oviposition. Pigments (protoporphyrin and biliverdin) are synthesized in the uterus and deposited by ciliated cells during the last 3 h before egg laying

(Zhao et al., 2006). Apart from providing physical protection to the chicken embryo, the eggshell functions effectively and efficiently in offering protection against bacterial ingression, providing a source of calcium and sites for respiration of the growing embryo. The mineral component and the organic matrix proteins interact to give the eggshell its physical and chemical protective function. Several genes and proteins are responsible for the eggshell formation. Identification and functional annotation of the uterine genes have the potential to serve as biological markers for genetic improvement of eggshell quality and egg self- life (Jonchere et al., 2010).

1.2.5 Vagina

The vagina is the caudal portion of the chicken oviduct and does not play a role in egg formation. However, this is where the outer cuticle forms and pigmentation occurs. The muscles of the vagina help to turn the egg longitudinally to the blunt end and push the egg out of the oviduct.

1.3 Functions of oviduct genes/proteins in the egg formation

1.3.1 Ovalbumin

Ovalbumin (OVL) describes a group of glycoproteins making up approximately 55% of total egg proteins and is found in the egg white. The presence of the ovum in the magnum increases OVL synthesis, and its expression is upregulated in chickens with high laying rate (Zhao et al., 2016). The ovalbumin protein family comprises three sequential homologous proteins: ovalbumin (OVAL), ovalbumin-related protein X (OVALX) and ovalbumin-related protein Y (OVALY). Their expressions in the chicken oviduct are regulated through estrogen, but the hormonal responsiveness is in the order of OV:Y: X=100:10:1 (Colbert et al., 1980). Progesterone induces OVL production in the magnum mucosa of laying hens (Joensuu et al., 1991), whereas OVAL expression decreases in molting birds (Zhao et al., 2016). The expression

of OVL is also increased in the magnum of juvenile, adult and molting hens treated with recombinant chicken growth hormone (Hrabia et al., 2014; Socha et al., 2017). OVALX is effective against Listeria monocytogenes and Salmonella enterica subspecies enterica, whereas OVAL is not. Since OVL is important as a constituent of the albumen, the antimicrobial activity adds specificity to the function of OVALX (Réhault-Godbert et al., 2013).

1.3.2 Avidin

Avidin is secreted by the goblet cells of the magnum. Additionally, avidin is expressed in the intestine and skeletal muscles of the developing embryo. Expression of avidin in the magnum is higher, 549-fold greater, in adult chickens than in juveniles (Jung et al., 2011). It has been reported that avidin in the oviduct scavenges unbound biotin and transfers it to albumen. Moreover, it is only when avidin becomes saturated by high dietary intakes that unbound biotin gets accumulated to yolk and plasma (White and Whitehead, 1987). Avidin production is induced in the magnum at a dose-dependent rate of progesterone administration (Joensuu et al., 1991).

1.3.3 Ovomucin

Ovomucin is a mucoprotein found in egg white and responsible for the gel-like property in fresh egg whites. Adult chickens show very high (4,931-fold) expression of ovomucoid in the magnum, more than juveniles (Jung et al., 2011). It represents the major allergen found in egg whites and is heat-stable, which accounts for around 3.5% of the total egg white protein. Because of its inhibitory effect on trypsin, it has been commercially available as a trypsin inhibitor, and can impart anti-viral (Tsuge et al., 1997) and anti-bacterial (Kobayashi et al., 2004) activities.

1.3.4 Ovoinhibitor

Ovoinhibitor (OIH) has been found to be highly expressed in the magnum and liver (Kim and Choi, 2014) and to a lesser amount in the uterus (Bourin et al., 2011). A study showed that

OIH expression increased in the liver of pre-laying hen and gradually decreased in matured birds (Kim and Choi, 2014). OIH has been shown to have serine protease inhibition activity (Matsushima, 1958) and provides defense against bacterial ingression of Bacillus spp. (Bourin et al., 2011). The inhibitory activity of OIH is more effective than ovomucoid (Feeney et al., 1963).

1.3.5 Ovostatin

One of the four proteinase inhibitors found in egg white is ovostatin (OVST) (Kitamoto et al., 1982). Because of its similar composition to plasma α2 macroglobulin, it has been earlier referred to as ovomacroglobulin. Unlike ovoinhibitor, its expression is limited to the glandular and luminal epithelium of the chicken oviduct and is induced by estrogen (Lim et al., 2011).

1.3.6 Ovotransferrin

Ovotransferrin (TF), previously known as conalbumin, is expressed predominantly in the magnum and the isthmus. This protein is found in the uterine fluid during the early mineralization stage (Gautron et al., 2001). The expression of ovotransferrin mRNA in the magnum is down-regulated with dietary corticosterone, but there is increased ovotransferrin protein in the egg white. Therefore, it can be used as a marker to estimate the exposure of the laying birds to environmental stress condition (Kim and Choi, 2014). Ovotransferrin has been reported to have an antimicrobial function (Baron et al., 2014; Giansanti et al., 2012).

1.3.7 Cystatin

Chicken cystatin is a cysteine proteinase inhibitor (Anastasi et al., 1983) and makes up 0.05% of the total egg white protein. Cystatin has antimycotic activity (Kolaczkowska et al., 2010) and is expressed in various tissues including lung, gizzard, brain, heart, muscle, liver, and oviduct of chicken. Its expression in magnum is not directly affected by estrogen (Colella et al., 1991).

1.3.8 Lysozyme

Lysozyme (LYZ) is an antimicrobial enzyme. The expression of LYZ in magnum is very much higher (37,557-fold) in adult chickens compared to juveniles (Jung et al., 2011). It makes up about 3.4% of the total egg white protein (Burley and Vadehra, 1989). The expression of lysozyme in magnum is massively high (37,557-fold) in adult chickens than in juveniles (Jung et al., 2011).

1.3.9 Collagen *X*

Collagenous fibrous proteins are the primary components of eggshell membranes. It gives the membrane its insoluble and stable nature. Collagen X (COL10A1) expression is localized in the tubular gland cells of the white isthmus (Wang et al., 2002). Microarray analysis has revealed that COL10A1 mRNA expression in the isthmus is 155-fold greater in the magnum and 111-fold greater in the uterus (Du et al., 2015).

1.3.10 Fibrillin-1

Fibrillin-1 (FBN1) is a microfibrillar glycoprotein rich in cysteine and is abundant in the extracellular matrix throughout the body. It is responsible for the elastic nature of chicken ESM and has 43 calcium-binding EGF-like repeats, which are the sites of nucleation for calcium deposition (Corson et al., 1993). Expression of FBN 1 mRNA in white isthmus is 2.5-fold higher than in the magnum, and 14-fold higher than in the uterus, respectively (Du et al., 2015). However, a proteomics study based on protein extraction with methanol failed to detect FBN1 protein from ESM (Rath et al., 2017). This indicated that FBN1 protein was highly insoluble in organic solvents (Du et al., 2015).

1.3.11 Cysteine-rich eggshell membrane protein

These eggshell membrane proteins (CREMPs) are very rich (13.8%) in cysteine, similar to fibrillin, and are significant contributors to the cysteine content in chicken ESM. The isthmus

shows the most robust expression of CREMP relative to other oviduct segments (Du et al., 2015). CREMP proteins also protect against microbial contamination in the egg (Kodali et al., 2011). The CREMP proteins contain multiple disulfide crosslinks that are formed through the actions of any of several enzymes, including sulphydryl oxidase, thioredoxin, protein disulfide isomerase (Kodali et al., 2011; Du et al., 2015).

1.3.12 Lysyl oxidases

The chicken lysyl oxidase (LOX) gene family consists of five gene members, namely lysyl oxidase (LOX) and lysyl oxidase homologue-1, -2, -3, and -4, (LOXL1, LOXL2, LOXL3, LOXL4). The members of this gene family contribute to the formation of crosslinks between eggshell membrane proteins (Harris et al., 1980). They are copper-dependent, and chickens fed a Copper-deficient diet have been shown to produce abnormal eggshell membranes (Harris et al., 1980). Among this enzyme family members, LOXL2, LOXL3 have been identified in the eggshell membrane by proteomics analysis (Sun et al., 2013). LOXL2 and LOXL3 are up-regulated in the isthmus relative to magnum and uterus respectively (Du et al., 2015), indicating that these homologues have a crucial role in forming crosslinks between collagen and ESM fibrillar proteins.

1.3.13 Quiescin q6 sulfhydryl oxidase 1

Quiescin Q6 sulphydryl oxidase 1 (QSOX1) is an enzyme having flavin-linked sulphydryl oxidase activity and protein disulfide isomerase activity. It catalyzes the formation of ESM proteins cross-linkages to impart the insolubility and stability of the ESM. Proteomic studies have confirmed the presence of this enzyme in ESM (Mann, 2008) and eggshell matrix (Mikšik et al., 2010). Du et al. (2015) reported an equivalent level of expression of QSOX1 in magnum, isthmus and uterus of chickens.

1.3.14 Thioredoxin

In chicken oviduct tissues, thioredoxin (TXN) is differentially expressed in the isthmus relative to both magnum and uterus (Du et al., 2015). As the name suggests, it helps to maintain the reduced state of protein thiol groups. It functions as an antioxidant by scavenging hydrogen peroxides. NADPH-dependent TXN has been identified as an activating factor to propitiate hormone-receptor binding (Housley et al., 1984).

1.3.15 Calbindin 1

Calbindin-1 (CALB1) is a calcium-binding protein, having a mass of 28 kDa found in the intestine, kidney, and oviduct of chickens and mammals. Expression and function of CALB1 are more evident and crucial in the oviduct (Nys et al., 1992) than in other tissues. During the calcification stage, its expression is highly increased in the uterus to accommodate the augmented need of calcium ions for mineralization of the eggshell (Jeong et al., 2012). Further, its expression and concentration in the shell glands are abruptly downregulated in the absence of an egg (Nys et al., 1992). Expression of CALB1 in uterine gland cells is related to abnormal eggshells due to intensified expression under such conditions (Arazi et al., 2009). Low dietary calcium intake leads to disrupted eggshells, and is associated with decreased uterine levels of CALB1 protein; however, this appears to be post-transcriptional, as calcium-deficiency has no effects on CALB1 mRNA levels (Nys et al., 1992). Though older birds have lower eggshell weights and densities, senescence does not affect uterine CALB1 (Yosefi et al., 2003). However, this is relatively undetectable during the molting stage and readjusts to normal levels when laying resumes. (Yosefi et al., 2003). Expression of CALB1 in eggshell gland is estrogen-dependent (Nys et al., 1992).

1.3.16 Cytochrome P450

Cytochrome P450 is a member of the superfamily of hemoproteins and a reducing

enzyme. Expression of CYP26A1, a member of the cytochrome P450 family, is upregulated in uterine tissue and is abundantly localized in the glandular epithelium of the uterus during the calcification stage, suggesting its supportive role in steroidogenesis and gene expression (Jeong et al., 2012; Brionne et al., 2014).

1.3.17 Proenkephalin

Proenkephalin (PENK) is a member of the opioid polypeptide hormone found in various mammals, rodents and avian species. Beside its dominance in the CNS, it is expressed in the oviducts in various species (Jeong et al., 2012). Two independent studies have reported increased expression of PENK mRNA in the shell gland during the calcification stage, indicating that PENK is required for eggshell (Jeong et al., 2012; Brionne et al., 2014). However, its mechanistic role in the uterus has not been established.

1.3.18 Acid phosphatase-1

Acid phosphatase-1 (ACP1) has the highest homology to mammalian ACP5 and is tartrate-resistant. It dephosphorylates phosphoproteins and enhances osteoclastic activity (Ek-Rylander et al., 1994). In the chicken oviduct, the ACP1 expression is induced when an egg is present in the magnum or the shell gland (Jeong et al., 2012). Along with the overexpression of SPP1 in the shell gland, it is believed to phosphorylate SPP1 and enhance eggshell mineralization.

1.3.19 Secreted phosphoprotein-1

Secreted phosphoprotein-1 (SPP1) gene, encoding for the protein osteopontin (OPN), is a potent inhibitor of calcification and helps in giving the shell its form and structure (Chien et al., 2008). Its secretion is induced by the mechanical distention of the uterine lumen, and unlike calbindin, is independent of calcium flux in the uterus (Lavelin et al., 1998). Expression of OPN in pre-laying chickens and hens with a premature expulsion of the egg (before calcification) is

null (Pines et al., 1995), which suggests that non-layers and molting birds lack OPN expression in the shell gland. OPN expression is weak in the magnum but high in the shell gland with the presence of an egg (Jeong et al., 2012). A localized expression is seen in the eggshell matrix and eggshell (Pines et al., 1995). Sometimes, secretion of osteopontin is irregular in the shell gland and correlates to abnormal eggshells like pimpled, corrugated, and cracked eggs (Arazi et al., 2009). Estrogen has been shown to suppress the expression of SPP1 in the oviduct (Lim et al., 2012b).

1.3.20 Regulator of calcineurin-1

The gene that regulates calcineurin-1 (RCAN1) in humans is annotated to regulate calcineurin-NFTA, signaling a cascade in a negative feedback mechanism (Minami et al., 2004). Calcineurin is a protein (serine/threonine) phosphatase activated by calcium and calmodulin. Its function is correlated with angiogenesis (Minami et al., 2004). RCAN1 regulates the expression of calcineurin to prevent these conditions. In the dearth of established mechanisms, its function is obscure in the chicken oviduct. However, an amplified expression level of RCAN1 transcripts in the shell gland during the eggshell calcification period is indicative of its signaling role on calcineurin, and thus shell calcification and oviposition (Jeong et al., 2012).

1.3.21 Ovocalyxin

It has been well established that the hard, calcified chicken eggshell is a result of the interaction between the organic matrix and inorganic minerals present in the uterine fluid. Ovocalyxin (OCX) - 32 and -36 are major constituents of the organic matrix (Jonchere et al., 2010; Brionne et al., 2014). Ovocalyxin-36 is an antimicrobial protein incorporated into the eggshell while ovocalyxin-32 is a major determinant of eggshell quality (Cordeiro et al., 2013). OCX-32 is secreted from the glandular epithelium of the shell gland and localized in the outermost cuticle layer of the eggshell, with higher expression in the distal oviduct (isthmus and

uterus) of chickens (Gautron et al., 2001; Jonchere et al., 2010). The OCX-36 is shell gland specific and is upregulated during calcification of eggshell (Jonchere et al., 2010; Brionne et al., 2014). This is localized mainly to the base of mammillary cores and the palisade layer of the shell (Hernández-Hernández et al., 2008). Expression of OCX-32 and -36 increases under the influence of growth hormones (Hrabia et al., 2014). Ovocalyxin-21 is another haplotype of the ovocalyxin gene family. It has been identified as uterus specific and expressed at stage I (initiation) and stage II (active growth) calcification (Hernández-Hernández et al., 2008). Presence of OCX-21 in stage I uterine fluid buffers the pH and create a conducive environment for rapid calcification during stage II.

1.3.22 Ovocleidin

Chicken ovocleidin 17 (OC-17) is expressed in the shell gland and has been identified as one of the eggshells soluble matrix proteins. It functions as an antimicrobial and biomineralization regulator (Hincke et al., 1995; Reyes-Grajeda et al., 2004). Reyes- Grajeda et al. (2004) observed a strong concentration-dependent correlation between OC- 17 and aggregation of calcite crystals. Further, OC-17 induces calcite crystal formation by binding to amorphous calcium carbonate in a catalytic manner (Freeman et al., 2010). Ovocleidin-116 is another member of the ovocleidin gene but is characterized as an insoluble eggshell matrix protein. Its distribution in the eggshell is similar to OC-17 (Hincke et al., 1999). Expression of OC-116 is higher in the uterus tissues compared to those in which the egg is expelled (Brionne et al., 2014).

1.3.23 Avian beta-defensins

Avian β defensins (AvBD) possesses antimicrobial activity in the chicken oviduct (Yacoub et al., 2015) and are incorporated into the eggshell membrane and eggshells (Mageed et al., 2009). Expression analysis of all 14 AvBD in the oviduct epithelium revealed that these

defensins are categorized into three groups, based on their expression levels under uninfected conditions: Group I (AvBD-4, -5, -9, -10, -11, and -12) with high expression levels; group II (AvBD-1, -3, -13, and -14) with moderate expression and group III (AvBD-2, -6, -7, and -8) with low expression (Ebers et al., 2009). Expression of AvBDs has been found in vaginal epithelium and these genes are upregulated by lipopolysaccharides (LPS) treatment in laying hens (Yoshimura et al., 2006; Mageed et al., 2008). Recently, a new member of defensin, called gallin, was identified in egg white and found to be over thousand times more highly expressed in the magnum than in the shell gland (Gong et al., 2010).

1.3.24 Solute carrier genes

Solute carrier (SLC) genes are mostly located in the cell membrane and code for membrane transport proteins. They transport glucose, electrolytes, and amino acids. The SLC superfamily is very wide and consists of 52 families. Since a large number of nutrients and electrolytes need to be transported from the liver and blood to the uterine environment for the proper composition of the chicken egg, SLC genes have an important role in the oviduct.

Currently, the identified SLC genes in chicken oviduct are SLC1A4, SLC13A2, SLC35B4, SLC4A5, SLC7A3, SLC41A2, SLC41A3, SLC25A30, SLC12A8, SLC16A2 and SLC4A9. SLC1A4 transports L-forms of serine, cysteine, alanine, and threonine. Glandular epithelium in the magnum expresses messenger RNA for this gene when the egg is present, allowing neutral amino acids to be incorporated into and support the developing egg mass (Lim et al., 2012a). Lim et al. (2012a) reported the expression of SLC4A5 (a co-transporter of sodium bicarbonate), SLC13A2 (dicarboxylate transporter), SLC35B4 (transporter of UDP-galactose) and SLC7A3 (transporter of cationic amino acids) genes in the magnum and shell gland, suggesting that these coded proteins play a significant role in incorporating nutrients in chicken eggs before they are laid. SLC41A2 and SLC41A3, associated with transportation of Mg-ion,

and SLC25A30, associated with the mitochondrial carrier, are differentially expressed in the shell gland during eggshell calcification (Brionne et al., 2014). Expression of SLC12A8, which is a pH regulator gene, is increased at the utero-vaginal junction (UVJ) in mated hens 24 h after natural mating compares to unmated layers. The increased expression of SLC12A8 at the UVJ suggests that mating and/or exposure of sperm is required for SLC12A8 up-regulation. (Atikuzzaman et al., 2015). Other SLC family members, SLC16A2 and SLC4A9 (pH regulators) are over-expressed in the UVJ after mating (Atikuzzaman et al., 2017).

1.3.25 Extracellular matrix remodeling and matrix metalloproteinases family

As the egg passes through the oviductal segments, a high amount of extra-cellular matrix remodeling (ECM) and cellular remodeling occur in the concomitant segment. Matrix metalloproteinases (MMP) are known to degrade the ECM for cellular proliferation, differentiation, migration, and apoptosis (Vu and Werb, 2000). MMP activities are induced by extracellular matrix metalloproteinase inducer (EMMPRIN) and inhibited by tissue inhibitors (TIMPs) (Mishra et al., 2012; Vu and Werb, 2000). In the chicken oviduct, the genes MMP-2 and -9 degrade gelatin and induce angiogenesis (Leśniak-Walentyn and Hrabia, 2016a). It was recently proposed that expression of MMPs in an age-specific and segment-specific manner in the avian oviduct is highly correlated to the undergoing cellular growth and differentiation for the required physiological needs (Leśniak-Walentyn and Hrabia, 2016a). In another study, involving forced molting of chicken, it was reported that MMP 9 had a further role in rejuvenating the oviduct (Leśniak-Walentyn and Hrabia, 2016b).

1.4 Role of steroid hormones in egg formation

Similar to the hormonal regulation of ovulation in mammals, the hypothalamus-pituitary-ovarian axis is the regulatory pathway of follicle development and ova formation in chickens. As in all vertebrates, the pineal gland in hens has photoreceptors which regulate the circadian

rhythm and the release of sex hormones from the pituitary gland. Photoreceptors in the hypothalamus are stimulated by the exposed photoperiod in a hen and GnRH, a peptide hormone, is released into. GnRH stimulates the synthesis and secretion of FSH and LH from the anterior pituitary. The GnIH inhibits synthesis and secretion of GnRH. The gonadorelin hormones travel through the blood-stream and reach the ovary where FSH stimulates follicular growth, while LH stimulates steroidogenesis by the follicles (Johnson and Bridgham, 2001). Some GnRH agonists such as long-acting leuprolide acetate, commonly used in birds for control of ovarian neoplasia, override the HPG axis by continuous GnRH like action and suppress the gonadal activity. The leuprolide acetate implants can have its suppressive action for 2-3 weeks on a dose-dependent manner (Mans and Pilny, 2014). Steroid hormones such as estradiol, progesterone, and growth hormones along with their receptors play a critical role in the development and functions of the chick oviduct (Kawashima et al., 1996). Concentrations of progesterone and estrogen receptors in the magnum and shell gland are regulated by chicken growth hormone, and their expression is decreased during the regression phase of induced molting (Hrabia et al., 2014; Socha et al., 2017). Estrogen induces expression of pleiotrophin, ovalbumin Y, and ovostatin, responsible for the development of the oviduct and the formation of the egg (Lim et al., 2011; Lee et al., 2012). Progesterone induces ovalbumin and avidin expression that codes for proteins of the egg white. Growth hormones regulate the expression of ovocalyxins, ovalbumin, and ovocleidin, whose coded proteins are important constituents of an egg (Hrabia et al., 2014; Socha et al., 2017).

1.5 RNA sequencing

The RNA-Sequencing (RNA-Seq) is growing as a powerful technique to quantify the presence of RNA transcripts in any given biological sample (Wang et al., 2009). It is based on the principle of high throughput sequencing which is exclusively being used to determine the expression profiles of genes in any cell or tissue at a certain time point. It can be used to study

the dynamics of transcriptomes in a tissue. While there is another common and cost-effective method of studying the transcript profiles such as microarray, the RNA-Seq technique have gained popularity in recent years because of its breakthrough discoveries. Microarray relies on the existing genomic knowledge of the species of interest, therefore is limited to analysis of pre-discovered genes only. However, the RNA-Seq can detect and discover any novel transcripts present in the sample. Moreover, it has advantages of higher accuracy, less background noise, requires less amount of RNA, able to determine gene isoforms, and quantify gene expression with over 8,000- fold changes.

1.6 Rationale of the study

1.6.1 Rationale for the study of magnum-specific genes

The egg-white (albumen) alone contributes more than 60 % to the total egg weight, and that is why the egg-white is of immense importance to the hatchery, and egg industry. Smaller eggs cannot make it to the hatcheries and also have low market value. Moreover, the consumers consider the egg as functional food because of the rich protein-albumen (Miranda et al., 2015). The food processing industry uses only the albumen portion of egg for its foaming and gelation properties (Alleoni, 2006). Fundamentally, the albumen is the principal source of nutrient for and barrier to pathogenic infestation of the developing embryo (Stevens, 1996). These perspectives necessitate an egg with qualitative and proportionate albumen in it. However, the genes/proteins and the biological pathways regulating the albumen synthesis, secretion, and deposition is still obscure. Therefore, transcriptomic analysis of the laying hens versus the non-laying hen's magnum, using RNA sequencing in this study, can reveal important molecules associated with the egg-white formation. The discovery of some novel genes can give insights into the process of egg-white formation and reveal molecular markers that can be used to improve the egg quality regarding size and safety.

1.6.2 Rationale for the study of uterus-specific genes

The uterus consists of the glandular and luminal epithelium whose secretions facilitate in eggshell biomineralization. Though the eggshell constitutes only about 10 % of the total egg weight, it has a crucial function of providing microbial and physical protection to the egg. It also provides a definite form to the egg, gaseous exchange, and plays a critical role in ensuring food safety and better hatchability.

Breeders have been continuously developing lines of long-life layers to achieve greater egg lay per bird (Bain et al., 2016) and some improvements have been achieved for better eggshell strength. However, further studies are required to understand the eggshell fabric and improve the eggshell quality. Irregularities in the formation of eggshell result in misshaped eggs, soft-shelled eggs, weak-shelled eggs and, sometimes other undesirable egg characteristics with calcite nodules and wrinkles. Approximately, 10% of the eggs produced in poultry farms are lost due to breakage of soft eggshells which accounts for huge economic loss to the egg industry ("Maintaining eggshell quality," 2008). So far, primary selection criteria for eggshell quality has been based on qualitative features such as eggshell specific gravity, breaking strength, and shell deformation (Hunton, 2005). However, selection based on quantitative traits with the knowledge of specific genes/proteins regulating the eggshell structure and quality is required to gain improvements in the eggshell quality in subsequent generations of layer-breeding stock (Hunton, 2005).

Eggshell is composed of 95% calcium carbonate which is transported in the form of calcium and bicarbonate ions from the bloodstream to the uterine cells, and ultimately into the uterine fluid in which the egg bathes (Jonchere et al., 2012). The genetic regulation of eggshell formation and biological pathways involved is highly complex and is not completely understood. Previous studies have reported the expression of several genes in the uterus, however, there is

little commonality in the reported genes from each study (Dunn et al., 2009b; Jonchere et al., 2010, 2012; Jeong et al., 2012; Brionne et al., 2014; Zhang et al., 2015). With the use of cutting-edge techniques and more precise time of sampling, each study reported some unpredicted genes and proposed their potential function in the eggshell mineralization. However, most of the earlier studies employed the long-established microarray technique that limits the detection of novel genes. Eggshell mineralization in chickens is the most efficient biological process regarding calcium mobilization and biomineralization. Therefore, with RNA sequencing, several novel genes and biological pathways associated with calcium-transport can be identified which will illustrate the existing knowledge on biomineralization process and help modulate the conditions in hens for better eggshell strength and quality.

1.7 Hypothesis

A few transcriptomic studies (Jeong et al., 2012, 2013; Wan et al., 2017) and some gene-specific studies (Sah and Mishra, 2018) have highlighted the importance of several genes and proteins involved in egg formation. However, the underlying molecular mechanisms and involvement of biological processes are still obscure. Therefore, we hypothesized that RNA sequencing (RNA-Seq) of a laying versus non-laying hen's oviduct could reveal novel genes and biological pathways having a significant role in albumen synthesis and eggshell formation.

1.8 Objectives

To test the hypothesis of this study, the following objectives were set;

- To identify the differentially expressed genes in the magnum of a laying versus non-laying and molting hens using RNA sequencing and real-time quantitative polymerase chain reaction (qRT-PCR).

- To identify the differentially expressed genes in the uterus of a laying versus non-laying and molting hens using RNA sequencing and qRT-PCR.

CHAPTER 2: MATERIALS AND METHODS

2.1 Animals and husbandry practices

All animal experimentations were carried out in accordance with the guidelines approved by the Institutional Animal Care and Use Committee of the University of Hawai‘i at Manoa (Approval no. 17-2605). The husbandry practices and tissue collection procedures were followed as per standard protocol of Small Animal Facility of College of Tropical Agriculture and Human Resources, University of Hawai‘i at Manoa. Hy-Line hens were brought from a commercial layer farm in Hawai‘i and acclimatized for two weeks in the research facility. All hens were housed individually in pens with deep-litter housing under the light regimen of 16-h of light (from 5-am to 9-pm) and 8-h of dark (from 9 pm to 5 am) with *ad libitum* access to feed and water. Hens were monitored daily, and their egg-laying times were recorded. Laying hens (35 weeks old; n=12) with more than 80% production rate, molting hens (around 60 weeks old; n=6), and non-laying hens (35-60 weeks old; n=6) were used for the research.

2.2 Tissue collection

Laying history and abdominal palpation were used to presume the presence of egg in the uterus, followed by post-mortem confirmation to determine the total time spent by the egg in the oviduct post-ovulation (p.o.). Molting hens were confirmed based on the laying history and necropsy observation for the presence of matured but unovulated follicles in the ovary. Non-laying hens were confirmed based on the absence of any growing follicles in the ovaries, and undeveloped oviduct. Magnum and uteri were collected from those laying hens where the egg was at albumen deposition stage (n=5) or at an active calcification stage (n=6), i.e. around 3 h and 15-20 h p.o., respectively. From the molting and non-laying hens, magnum and uteri tissues were collected at similar time-points parallel to sampling of laying hens. The albumen is

deposited when the egg is in the magnum, whereas, the eggshell biomineralization starts when the egg is in the uterus. Concurrently, the expression of the genes involved in albumen deposition, and eggshell calcification is supposed to be higher during 3-h p.o. and 15-20 h p.o., respectively. For RNA analysis, portions of the oviductal tissues were collected and snap- frozen immediately in liquid nitrogen, then stored at -80°C until analysis.

2.3 Total RNA isolation and quality control

Total RNA was isolated from frozen tissues (50-100 mg) using TRIzol reagent (Invitrogen, Carlsbad, CA) according to the manufacturer's instructions. The RNA isolation protocol employed the following steps: tissue homogenization, RNA extraction using chloroform, phase separation, RNA precipitation, RNA pellet washing, drying, and resuspension. First, the collected tissue chunks were kept in individual tubes containing 1 mL of TRIzol reagent and homogenized using Kinematic Polytron™ homogenizer (Kinematica USA, Bohemia, NY) for 20-30 seconds or until complete homogenization of the tissue. The tissue homogenate was transferred to a new microcentrifuge tube and allowed to stand at room temperature for 5 minutes. Then, 0.2 mL (per 1 mL of Trizol used) of chloroform was added to the homogenate, vortexed briefly to mix, and allowed to react for 5 minutes. The homogenate solution was centrifuged for 15 mins @ 9000× g at 4^0 C. Following centrifugation; the homogenate separates into three distinct layers: an upper aqueous layer containing the RNA, the bottom organic phase containing protein and the middle interface with DNA. The upper clear liquid was pipetted and transferred to a new microcentrifuge tube. The RNA was precipitated by adding 0.5 mL of isopropanol to the aqueous solution, mixing, allowing to settle for 5 minutes, and centrifuging for 10 mins @ 9000x g at 4^0 C. The supernatant from the RNA pellet was discarded and 1 mL of 75% ethanol was used to wash the pellet to remove any salts present, and then centrifugation for 5 mins @ 13000× g at 4 ^{0}C. The supernatant ethanol was discarded, and

the RNA pellet was air-dried by inverting the tubes for 10 minutes. The RNA pellet was resuspended in nuclease-free water (Thermo Scientific, Waltham, MA) and incubate on a heating plate at 60 ^{0}C for 10 minutes.

The concentration of total RNA was determined using NanoPhotometer® P330 (IMPLEN, Los Angeles, CA). RNA quality was determined with the Agilent 2100 Bioanalyzer (Agilent Technologies, Massy, France). The samples with an RNA integrity number (RIN) > 8.5 (represented in figure 2) were further used for RNA-Sequencing, and quantitative real-time PCR. The RNA samples were stored in ultra-low temperature freezer at -80 ^{0}C until further use.

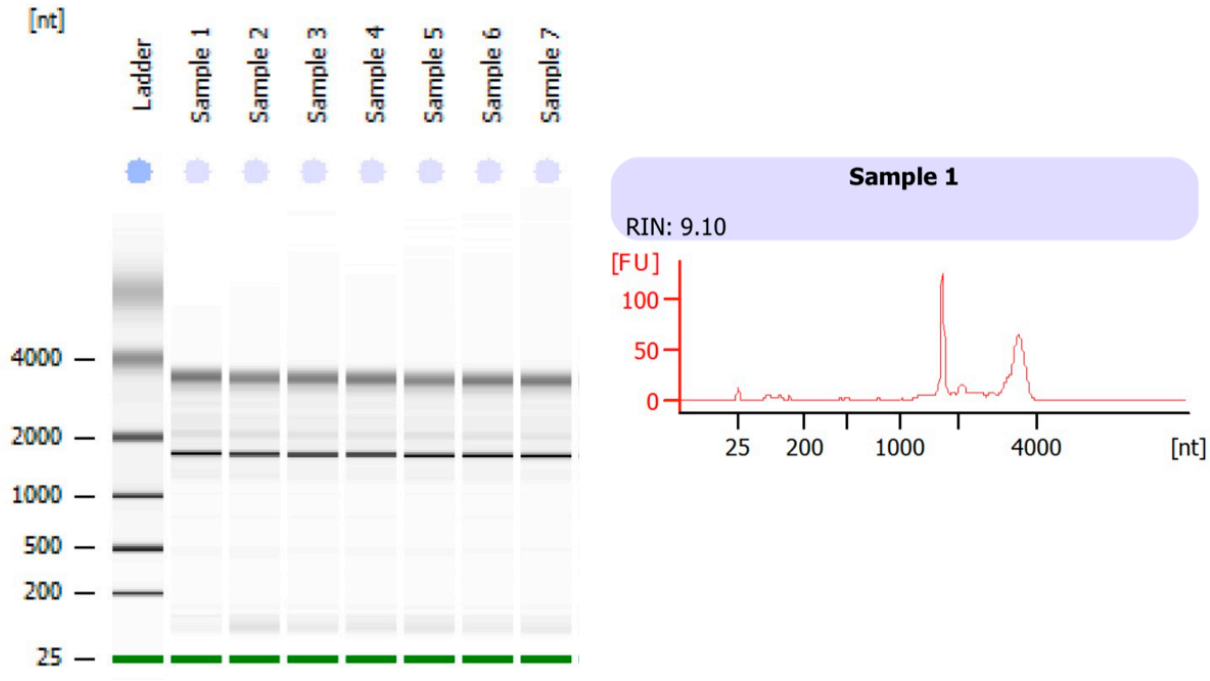

Figure 2 A representative image showing electrophoresis run of a good quality RNA sample measured using 2100 Agilent Bioanalyzer.

2.4 Library preparation and sequencing

RNA-Seq libraries were prepared and sequenced at the University of Hawai‘i Cancer Center Genomics and Bioinformatics Shared Resource (UHCC GBSR) facility. A TruSeq

Stranded mRNA kit (Illumina, San Diego, CA) was used to prepare the RNA-Seq libraries from total RNA samples extracted from laying hens at 15-20 h p.o. (n=3), and non-laying hens (n=3) uteri. Libraries were prepared according to the manufacturer's protocol without any modification. Briefly, poly-A RNA fraction, containing mRNAs and certain non-coding RNAs, was enriched from 500 ng of total RNA in two rounds of purification using Poly-T oligo magnetic beads. RNA was eluted using Elute, Prime, Fragment Mix followed by 8-minute fragmentation at 94°C. Fragmented RNA samples were subsequently used for cDNA first- and second-strand synthesis, then the blunt-ended dsDNAs were separated using AMPure XP beads (Beckman Coulter, Brea, CA) from the unincorporated nucleotides and enzymes. cDNAs were then adenylated at their 3' ends followed by ligation of indexing adapters, specific for each sample. The DNA fragments with adapter molecules on both ends were enriched with 15 cycles of PCR followed by two rounds of purification on AMPure XP beads. The size and quality of the libraries were assessed in a High Sensitivity DNA Bioanalyzer assay (Agilent Technologies, Massy, France). Next, libraries were quantified by qPCR using KAPA Library Quantification Kit (KAPA Biosystems, Boston, Massachusetts) and were normalized to the concentration of 4 nM. Libraries were pooled and denatured using freshly prepared 0.2N NaOH followed by further dilution with HT1 buffer to obtain a final concentration of 1.8 pM. As a sequencing control, the library pool was spiked-in with 1% (v/v) of 1.8 pM denatured PhiX library and loaded onto the reagent cartridge. The sequencing run was performed with NextSeq 500 (Illumina, San Diego, CA), in single-end mode with a read length of 1×76bp. Illumina BaseSpace-created FASTQ files were used for further analysis.

2.5 RNA sequence analysis

Data analysis of the RNA sequences was done at the University of Hawai'i John A. Burns School of Medicine Bioinformatics core. Single-end reads in the FASTQ format were

explored using FastQC (Babraham Institute, Cambridge, UK) and cleaned using Prinseq, a Perl script (Schmieder and Edwards, 2011). The cleaning procedure included trimming low quality reads from both 3' and 5' ends until a base pair of Phred quality score of 30 (99.9% accurate) or greater was found and filtering out reads having a mean quality score less than 30 and length below 30 nucleotides. Cleaned reads were aligned against chicken reference genome Galgal 5.0 using in Array Studio (version10; OmicSoft, Cary, NC; http://www.omicsoft.com/array-studio). Differential gene expression analysis in layers with respect to non-layers groups was performed by the DESeq2 algorithm (Love et al., 2014) as implemented in Array Studio. The genes with at least two-fold change (FC) and Benjamini and Hochberg q-value < 0.05 were called differentially expressed (DE) in the uterus while, FC greater than 3.0 was cut off value for DE in magnum. The DE genes with higher expression in laying hens compared to non-laying hens were referred as up-regulated genes in laying hens and vice-versa. The pipeline used for RNA-Seq analyses is shown in Figure 3.

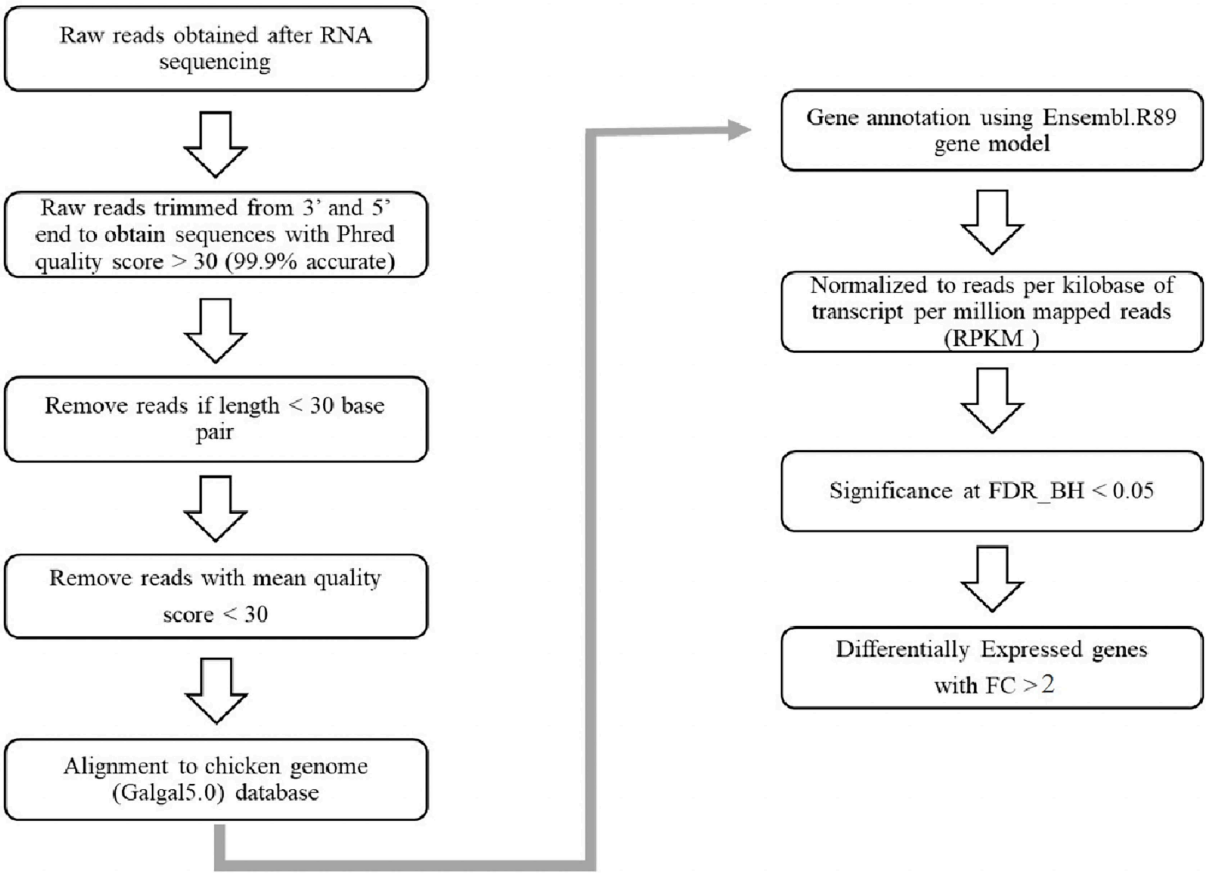

Figure 3 Processing of RNA-Seq data to obtain high-quality non-redundant transcriptomes.

2.6 Functional annotation and Gene Ontology enrichment analysis

An open web source, Database for Annotation Visualization and Integrated Discovery (DAVID) system (https://david.ncifcrf.gov/home.jsp), was used to gain insight into the various Gene Ontology (GO) terms of the upregulated genes in layers. The official gene symbol of the upregulated genes was uploaded to the functional annotation tool in the DAVID system, and the chicken was selected as the reference genome. The genes that matched up with the genes in DAVID were annotated into three GO terms; biological process (BP), cellular component (CC), and molecular function (MF). All the GO terms were considered enriched at a modified P-value < 0.05 and threshold gene count of 2.

2.7 Pathways analysis

The ingenuity pathway analysis (IPA) is a human genome-based powerful search tool with several advanced functions that allows insightful data analysis and interpretation. The differentially expressed genes (DEGs) were subjected to the Ingenuity Pathway Analysis (IPA; QIAGEN Inc., https://www.qiagenbioinformatics.com/products/ingenuity-pathway-analysis; Kramer et al. 2014) to gain insights into the canonical pathways and network discovery. Credible conclusions were derived from the pathways, networks, and functions since the IPA makes the annotation based on the human genome.

We also analyzed the pathways enrichment for the upregulated genes in layers using the Kyoto Encyclopedia of Genes and Genomes (KEGG) (http://www.genome.jp/kegg). The official gene symbol of the upregulated genes was uploaded to the functional annotation tool in the DAVID system, and the chicken was selected as the reference genome. The enrichment parameters were set to a threshold gene count of 2 and a modified Fisher Exact P-value < 0.05. The over-represented KEGG pathways terms were considered enriched KEGG pathways.

2.8 Complementary DNA synthesis (cDNA)

The synthesis of the first-strand cDNA was performed by reverse transcription of 1 µg total RNA (20 µL reaction of RT mixture) using High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Foster City, CA). The RNA samples were first diluted with nuclease-free water to standardized them to a concentration of 1 µg per 10 µL. Then, 10 µL of reverse transcriptase (RT) mix was prepared by using 2 µL of 10X RT Buffer, 0.8 µL of 25X dNTP (100nM), 2 µL of 10X RT Random Primer, and 1 µL of Multiscribe Reverse Transcriptase enzyme (Applied Biosystems, Foster City, CA).

The RNA sample (10 µL) and RT mix (10 µL) were mixed in a microcentrifuge tube and incubated into a thermal cycler for the synthesis of single-stranded cDNA. The run condition of

the thermal cycler was 25^0C for 10 minutes, 37^0C for 120 minutes, 85^0C for 5 minutes, and 4^0C for infinity. The newly synthesized cDNA (20 µL) was diluted (25X) with 480 µL of nuclease-free water. Finally, the cDNAs were store at -20^0C until qPCR assay.

2.9 Quantitative real-time PCR (qPCR) assay

The qPCR assay was done in 10 µL reaction mixture containing 3 µL of cDNA and 7 µL of PCR mix on a StepOne™ Plus real-time PCR system (Applied Biosystems, Foster City, CA). The PCR mix was prepared by adding 5 µL of PowerUp SYBR Green Master Mix (Applied Biosystems, Foster City, CA) and 1 µL each of forward and reverse primers (Tables 2, 3, and 4) specific to the gene target. Primer pairs (forward and reverse) specific to each candidate genes were designed using NCBI primer blast tool. The PCR mix and cDNA samples were loaded into the wells of a 96-well microtiter plate and sealed with clear optical adhesive films (Applied Biosystems, Foster City, CA). A PCR run template file was designed using the StepOne™ Plus software v2.3 (Applied Biosystems, Foster City, CA) in accordance with the reagents used and the samples loaded. Then the PCR plate was inserted on to the StepOne™ Plus machine. The amplification conditions were 50^0C for 2 minutes (hold), 95^0C for 2 minutes (hold), followed by 40 repeat cycles of 95^0C for 15 seconds (denaturation), 60^0C for 15 seconds (annealing), and 72^0C for 1 minutes (extension). A melting curve was also generated to confirm the sequence-specific PCR products. After the run was completed, the data was downloaded, and the PCR plates were stored at 4^0C until validated for target specificity by gel electrophoresis.

Table 2 Primers used to quantify the expression of magnum-specific candidate genes by qPCR.

Gene	Accession no.	Sequences	Amplicon (bp)
AVD	NM_205320.1	F: TCAAAGAGTCACCACTGCAT R: TTGATGCCGACCCTGGTA	223
TMPRSS9	XM_425880.5	F: GCTTTGATATTTACAGTGACCCC R: AAGGGTGCTTGTAGATACGG	111
MMP1	XM_417176.4	F: CTCTGAAAGTCTGGAGCAGT R: ACAAGTTGTAGCCTCTTCCA	228
ACE	NM_001167732.1	F: CAACAAGGAAGTCATGCTGG R: TCTGAGAGGAGGGTGTTGTA	195
ATG10	XM_015280713.1	F: GAACTACTAAGGGGCAGAAG R: GCAGGAAACATGGAACTAGA	125
GNRHR	NM_001012609.1	F: CACGAGACCCTCTACAACAT R: GTCCCTGGAGGAGAAGAGG	135
AvBD11	NM_001001779.1	F: GTTGGCTACCATGGGTACTG R: AGTCTGATGTAGTGTCTACGC	112
RLN3	NM_001113200.1	F: GCGGCCGATTCTTCTCAAG R: GTCATCTGCCATAGACTCGT	168
MMP9	NM_204667.1	F: TCAAGGTGTGGAGTGATGTC R: CTTCACCTCTAAGCCGGTTC	224
MELTF	NM_205207.2	F: GCTCCAACATCACCATCAAC R: TCACTAACAGCTTTCGGGAG	151
GPX3	NM_001163232.2	F: GAGGGAGAAGGTGAAATGCT R: CCCAGCTCATTTTGTAGTGC	192
CGN	NM_001347391.1	F: GAGTGGAGGACATCGAACAG R: AGGTCATTGATGAAGCGGAT	104
PROC	XM_015276809.1	F: AGCATAAAGCTTGTCAGAAGT R: TGTAAAATATCGAGGCATGG	171
PSPH	NM_001252272.1	F: CTCGAGCAGCAGCAG R: CTCTGCGGGTCATCTCT	204
PHGDH	XM_422226.5	F: CTTTTGGCATGAAGACCATC R: CTCCTTCATCCACAATGCC	234
ASNS	NM_001030977.1	F: CGGCACGGGGTAACTC R: TCAAAACGAAATGCATCAGG	132
PSAT1	XM_424846.5	F: CCAGATGCATCGTATGTGTA R: CACACCAAACTTGGAAACAT	150
MMP10	NM_182973.2	F: GGCAACAAGTATTGGAGTTTTGATG	202

		R: TGCTCTTCATTTTACGGACCA	
CAPN2	NM_205080.1	F: GACCTGCTAGATAACGATGG	
		R: ATACGAGTTCATGGTTCCAG	132
REN	XM_015299118.1	F: CAGGATGTGGTCATGGTATC	
		R: GGCTGTAGTAGACAGAGAAC	211

*F=Forward; R= Reverse

Table 3 Primers used to quantify the expression of select members of SLCs in the magnum of hens by qPCR.

Gene	Accession no.	Sequences	Amplicon (bp)
SLC6A17	XM_015299148.2	F: TCAACTTCTTCACCTCCGTC	105
		R: TCTTCTCAGCATTCTCCACC	
SLC7A11	XM_426289.5	F: CATTGTTTTGCACCCTCTCA	156
		R: AAAGGACGAGGCATATCAGG	
SLC7A7	XM_015282844.2	F: GAAAACCTCAGAGCTCCCTT	148
		R: GAGGTAAATTCCTCTCGGGG	
SLC9A2	NM_001285935.2	F: CGGTGGAGTACTGATTGGAA	168
		R: CAAGCTGTAATCGCCATGAT	
SLC10A4	XM_001233593.5	F: TTTCCTCCTCGCCCTCAT	136
		R: GATGATGCTGAGGGTCATGT	
SLC16A12	XM_025152017.1	F: GGCTAACAGACAGAAGGTGT	128
		R: TGAGAAAGGCACAAGCAAAG	
SLC16A5	XM_025141795.1	F: CTCACATTCCAGGTGCTGAT	138
		R: ATATCCACCAGGAGACCAGT	
SLC17A9	NM_001006292.1	F: GACAAGAAGCAGTCAGGAGT	103
		R: CCTTCTCACCCCCTATTTGAT	
SLC1A4	XM_001232899.5	F: CCGTCGCTCTCGGCTT	139
		R: AACAGGTTTCTGACGAGGTC	
SLC22A18	XM_421021.5	F: CCTGACAGCTCCTTCTGAAC	127
		R: CATGGGAACAAAAATGCCGA	
SLC22A3	XM_419620.6	F: TGTGCTCAAGTTTGTGTGAC	109
		R: GCTGCGAGAACACTGAAAAT	
SLC25A4	NM_001006443.1	F: ACACAGCCAAGGGTATGTTG	127
		R: ATCCTACGTCGCACAGTATC	
SLC25A48	XM_025154931.1	F: ACTTTTTCTCAGCTGTAGCTCT	149
		R: AACGAGTCTTGACGGTGTCC	

SLC26A4	XM_425419.6	F: TGTTGTAGAACCAGAGGGTG R: ATCCCACTGTGGATTTGAGG	101
SLC37A2	XM_015298088.2	F: ATGCTGTTCCTGTACAACCA R: GGACTCATGGGTTCCCAAAT	141
SLC51B	XM_025153901.1	F: TATGACCACACGGCATACAA R: TGGATCTTCCTTTTCGGTCT	106
SLCO2A1	NM_001198927.2	F: TCCCAGACAGGTGATCTACA R: AAAGGAGATGAGGAAGACGG	130

*F=Forward; R= Reverse

Three house-keeping genes Glyceraldehyde 3-phosphate dehydrogenase (GAPDH), Beta-actin (B-actin), and TATA-Box Binding Protein (TBP) were analyzed in triplicates in each hen of the experimental groups to determine the most stable house-keeping gene in uterine tissues. The target genes were analyzed in duplicates and expression level was determined using cycle threshold (Ct) values following standard curve method after normalization with TBP. Fold change for each gene was calculated using the $2^{-\Delta\Delta Ct}$ method. The average of the Ct values for the housekeeping gene and the gene being tested in the experimental and control conditions were taken; Tested Experimental (TE), Tested Control (TC), Housekeeping Gene Experimental (HE), and Housekeeping Gene Control (HC). Then, the differences between TE and HE (i.e. ΔCtE = TE-HE) and TC and HC (ΔCtC = TC-HC) were calculated. Finally, the difference between ΔCTE and ΔCTC (i.e. ΔCTE-ΔCTC) was calculated which gave the Double Delta Ct value (ΔΔCt). Data for fold change were presented as a mean ± standard error.

Table 4 Primers used to quantify the expression of uterus-specific candidate genes by qPCR.

Gene	Accession no.	Primer Sequence	Amplicon (bp)
MEPE	NM_204569.1	F: CCTGGACAGCACCAGATACT R: ATGGCTGCTTCCAACACCTT	201
OTOP 2	XM_015295462.1	F: GAGCAAGCAATTGCCCAAAATG R: CGTGAGACGTGGTATGAGGA	247
ATP2B2	XM_015293478.1	F: TGTGCACTATGTTCATGACTGA R: CACTCCATTCACCACTTCCC	181
GNRHR	NM_001012609.1	F: CACGAGACCCTCTACAACAT R: GTCCCTGGAGGAGAAGAGG	135
OSTN	XM_015291403.1	F: GCAAACAGAGGAAAGTGGTTG R: AGAAGATTTTGCAGGAGGTCAA	218
ATP1A1	NM_205521.1	F: TGAGTCGGGGTTTGACTACT R: CGATCCACAACAGGAGTGAG	144
ATP2A3	NM_204891.1	F: CTCATCTTCCAAGTGACCCC R: TACTGTCCTGAGAATCCCCTC	135
CALCB	NM_001113708.1	F: GCACCGCCGAGAGGAAA R: TCGTAATCACTGAGCGTCAC	153
OTOP 3	XM_420115.4	F: GGAGCTCTGTTGCTGTTTCT R: CTGTGGGTCACCTTTCACAC	189
CAMK1D	XM_003640331.3	F: TGGATTGCAGGTGATACAGC R: AGTGGATGGAGACATACAATCTT	246
STC2	XM_414534.5	F: ACATCCGGAGGTCTAGCTG R: TGGTTCGAGCTTGTTCTACC	180
ATP1B1	NM_205520.4	F: CCCCCTACGTTTACCCGA R: AGGATCTTAAACCAGCTGCC	204
ATP2C2	XM_004944264.2	F: ACTTGGCAGCAGGTAACAC R: CACTCCTGGCTCTCCTCTT	220

*F=Forward; R= Reverse

2.10 DNA gel electrophoresis

The specificity of PCR products was verified using 2% agarose gel electrophoresis. First, 2 gm of agarose was weighed out in a volumetric flask and 100 mL of 1X Tris-borate-EDTA (TBE) buffer. Then, the solution was microwaved briefly for 1-2 minutes in pulses of 30 seconds until complete dissolution of agarose (clear solution was visible). About 2-3 µL of 1% ethidium

bromide (EtBr) was added to the melted agarose solution. EtBr binds to the DNA and allows visualization of the DNA under ultraviolet (UV) light. The agarose solution was let to cool down to about 50°C for 5 mins and poured into a gel tray. A comb was inserted to create the wells and allowed to solidify for 20-30 minutes. Meanwhile, PCR products were prepared for electrophoresis.

Loading samples were prepared in the ration of 5:1 by adding 5 µL of PCR product and 1µL of loading buffer (Omega Bio-tek Inc., Norcross, GA). The molecular weight (MW) of the samples was compared with a DNA molecular weight marker (100bp, VWR, Radnor, PA) to determine the specific sizes of the PCR products. After casting the gel, it was transferred to the gel box, and 1X TBE was poured to submerge the gel. Next, 5 µL each of samples and the MW marker was filled in the gel wells. After complete loading of the gel, the run was initiated for 1 hour at 100 volts.

Following completion of the run, the gel was counterstained with 1X TBE containing EtBr for 20 minutes. Then, the gel was washed 3X times in tap water. Finally, the gels were visualized for the presence of DNA bands. The MW of the PCR products were noted from the gels and matched up with that of the respective genes. Samples with DNA bands of MW similar to primers used were confirmed as specific amplification by qPCR.

2.11 Data availability

The RNA-Seq datasets generated in the current study are available in the Gene Expression Omnibus (GEO) repository and can be accessed with the accession number GSE114103.

2.12 Statistics

Data of the RNA-Seq were analyzed in Array Studio. Differential expression of genes was declared at fold change (FC) greater than 2 in the magnum, and FC > 3 in the uterus in addition to a Benjamini and Hochberg q-value < 0.05. The fold-change values calculated for qPCR results were analyzed using Statistical Analysis Software. Values were subjected to one-way analysis of variance (ANOVA) followed by Tukey-Kramer test for multiple mean comparison to determine differences and significance were declared at p-value < 0.05 on a SAS platform.

CHAPTER 3: RESULTS

3.1 Differentially expressed genes in the magnum

3.1.1 RNA-Seq results and identification of DEGs in magnum

Raw sequencing reads in FASTQ format from replicated RNA-Seq libraries were obtained, and their qualities were checked using FastQC. There was an average of 30.5 M and 33.4 M original raw reads in layers and non-layers, respectively. After trimming and filtration, more than 97 % of input reads from both layers and non-layers were retained as excellent quality sequences (Table 5). Mapping results to the chicken genome database showed that an average of 97.42 % of the retained reads from layers and 87.88 % from non-layers was uniquely mapped (Table 6). A total of 19,152 gene transcripts were annotated from Ensembl alignment which represented 50.24% of the chicken genome assembly. DESeq2 analysis showed 540 genes were differentially expressed between laying and non-laying hens (Data available at Gene Expression Omnibus with accession no GSE114103).

Table 5 Filtration summary of RNA-Seq raw reads in the magnum of laying (3 h p.o.) and non-laying hens.

	Non-layers			Layers		
Observations	Library 1	Library 2	Library3	Library 1	Library 2	Library3
Input sequences	33,417,905	33,413,560	33,270,562	29,400,849	31,761,752	30,356,773
Input bases	2,523,961,191	2,523,448,331	2,512,432,615	2,220,156,836	2,398,309,598	2,292,629,021
Input mean length	75.53	75.52	75.52	75.51	75.51	75.52
Good sequences	32,462,423	32,536,676	32,261,912	28,796,956	31,027,354	29,710,581
Good bases	2,447,911,639	2,453,686,361	2,432,291,021	2,171,950,684	2,339,859,559	2,241,038,894
Good mean length	75.41	75.41	75.39	75.42	75.41	75.43
Bad sequences	955,482	876,884	1,008,650	603,893	734,398	646,192
Bad bases	72,068,118	66,124,816	76,012,479	45,556,882	55,398,607	48,735,352
Bad mean length	75.43	75.41	75.36	75.44	75.43	75.42
trim_qual_left	3634	3253	5545	1525	2138	2189
min_len	4172	5295	4803	3645	8480	3012
min_qual_mean	947676	868336	998302	598723	723780	640991

Among the DEGs, 457 genes were officially characterized while the rest were novel transcripts without any annotation. The number of up-regulated and down-regulated genes in laying hens (3 h p.o.) were 152 and 388, respectively when compared to the non-laying hens. A list of the top 30 over-expressed and under-expressed genes with their relative fold change in the magnum of laying hens are presented in Table 7 and Table 8, respectively. A visual representation of the 30 most up-regulated and down-regulated genes in layers shown as a heatmap image in Figure 4.

Table 6 Summary of magnum RNA-Seq data mapping to the chicken genome (Galgal5.0).

cDNA library	Reads (pre-filter)	Reads (post-filter)	Uniquely Mapped reads
Non-layer 1	33,417,905	32462423	27246689 (88.10%)
Non-layer 2	33,413,560	32536676	28721116 (90.80%)
Non-layer 3	33,270,562	32261912	27655790 (84.75%)
Layer 1	29,400,849	28796956	32462423 (94.61%)
Layer 2	31,761,752	31027354	32536676 (92.56%)
Layer 3	30,356,773	29710581	32261912 (93.08%)

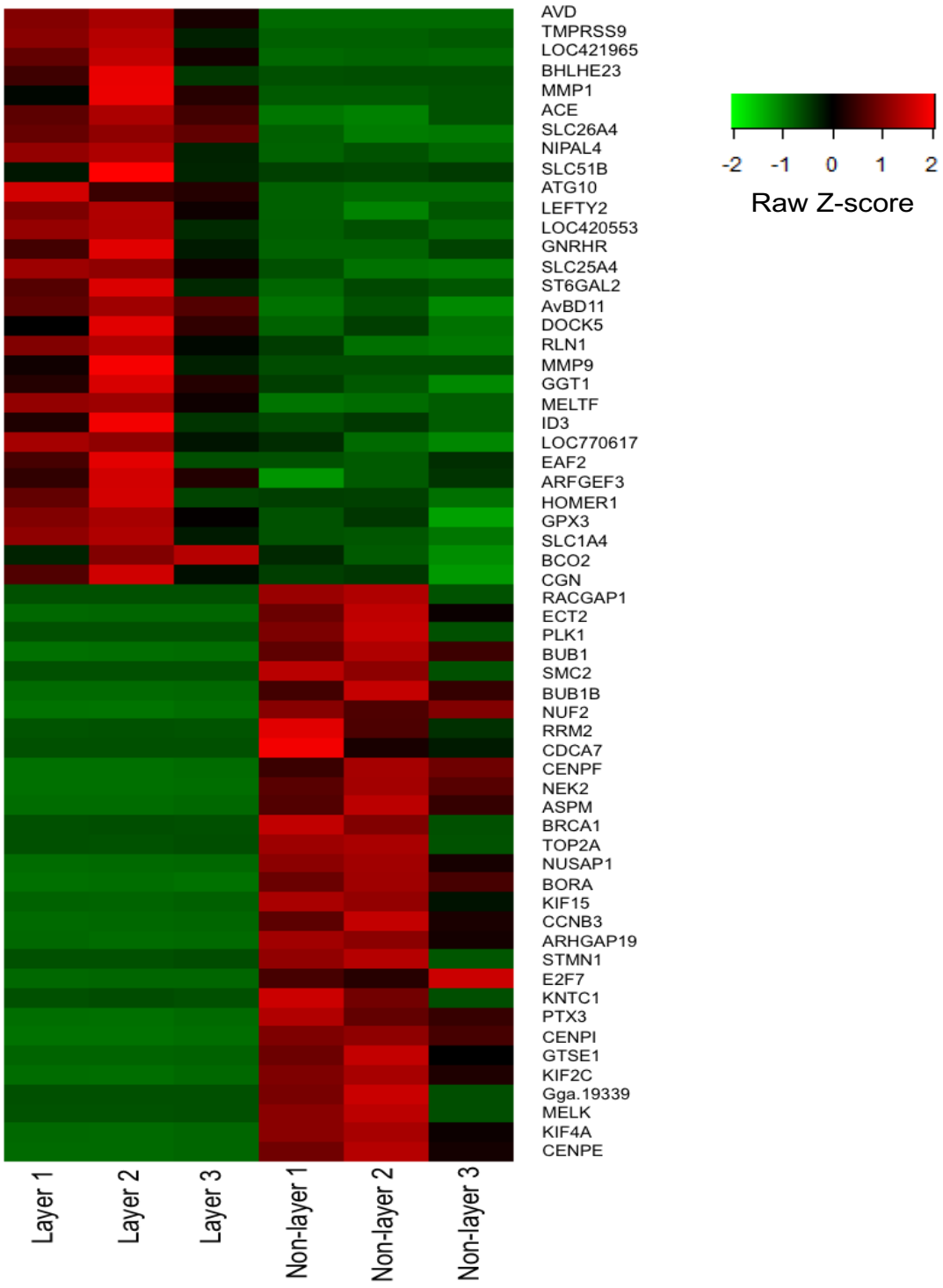

Figure 4 Heat-map of the 30 up-and down-regulated genes in the magnum of laying hens. Extremes of red and green colors indicate the highest up- and down-regulated genes, respectively, in laying and non-laying hens. The color contrasts depict the difference in the gene expression between layers and non-layers.

Table 7 The 30 most up-regulated genes in the magnum of laying hens.

Gene Name	Gene Description	Fold Change
AVD	avidin	250.6824
TMPRSS9	transmembrane protease, serine 9	33.0611
LOC421965	LOC421965	29.9439
BHLHE23	basic helix-loop-helix family member e23	17.2940
MMP1	matrix metallopeptidase 1	16.0790
ACE	angiotensin converting enzyme	14.8313
SLC26A4	solute carrier family 26 member 4	9.7876
NIPAL4	NIPA like domain containing 4	8.8105
SLC51B	solute carrier family 51 beta subunit	8.7190
ATG10	autophagy related 10	8.6403
LEFTY2	left-right determination factor 1	8.5433
LOC420553	LOC420553	8.4146
GNRHR	gonadotropin releasing hormone receptor	7.7644
SLC25A4	solute carrier family 25 member 4	7.5906
ST6GAL2	ST6 beta-galactoside alpha-2,6-sialyltransferase 2	7.5662
AvBD11	avian beta defensin 11	7.5066
DOCK5	DOCK5	7.4964
RLN1	relaxin	7.4939
MMP9	matrix metallopeptidase 9	7.4666
GGT1	gamma-glutamyltransferase 1	7.0938
MELTF	melanotransferrin	6.9221
ID3	inhibitor of DNA binding 1, HLH protein	6.5950
LOC770617	LOC770617	6.4296
EAF2	ELL associated factor 2	6.0836
ARFGEF3	ARFGEF family member 3	5.8841
HOMER1	homer scaffolding protein 1	5.6404
GPX3	glutathione peroxidase 3	5.6046
SLC1A4	solute carrier family 1 member 4	5.5812
BCO2	beta-carotene oxygenase 2	5.4316
CGN	cingulin	5.3925

Table 8 Top 30 down-regulated genes in the magnum of laying hens.

Gene Name	Gene Description	Fold Change
RACGAP1	Rac GTPase activating protein 1	16.6262
ECT2	epithelial cell transforming 2	14.9228
PLK1	polo like kinase 1	14.0534
BUB1	BUB1 mitotic checkpoint serine/threonine kinase	13.9712
SMC2	structural maintenance of chromosomes 2	13.8266
BUB1B	BUB1 mitotic checkpoint serine/threonine kinase B	13.7985
NUF2	NUF2, NDC80 kinetochore complex component	13.3569
RRM2	ribonucleotide reductase regulatory subunit M2	12.9268
CDCA7	cell division cycle associated 7	12.8785
CENPF	centromere protein F	12.7394
NEK2	NIMA related kinase 2	12.5734
ASPM	abnormal spindle microtubule assembly	12.2945
BRCA1	BRCA1(Breast cancer)	12.2056
TOP2A	topoisomerase (DNA) II alpha	11.5891
NUSAP1	nucleolar and spindle associated protein 1	11.1474
BORA	bora, aurora kinase A activator	11.0740
KIF15	kinesin family member 15	10.6809
CCNB3	CCNB3 (cyclin B3)	10.6117
ARHGAP19	ARHGAP19	10.4602
STMN1	stathmin 1	10.4540
E2F7	E2F transcription factor 7	10.4150
KNTC1	kinetochore associated 1	10.0559
PTX3	pentraxin 3	9.96580
CENPI	centromere protein I	9.86620
GTSE1	G2 and S-phase expressed 1	9.73060
KIF2C	kinesin family member 2C	9.64650
Gga.19339	family with sequence similarity 72, member A	9.57600
MELK	maternal embryonic leucine zipper kinase	9.47710
KIF4A	kinesin family member 4A	9.29000
CENPE	centromere protein E	9.28690

3.1.2 Functional annotation and pathways enrichment analysis of DEGs in magnum

A total of 121 up-regulated genes were uploaded for functional annotation in the DAVID system and results showed 119 genes were annotated. About 85 genes were recognized in biological process, and three processes, L-serine biosynthetic process, regulation of immune system process, and proline transport were enriched (Figure 5a). The molecular function had

only one enriched GO term, i.e., transporter activity with 83 genes recognized (Figure 5b) while cellular component contained 4 enriched GO terms of the 90 identified genes (Figure 5c). We also analyzed the pathways enrichment for the upregulated genes in layers using KEGG pathways as incorporated in DAVID system. Glycine, serine and threonine was the only pathway to be enriched for upregulated genes.

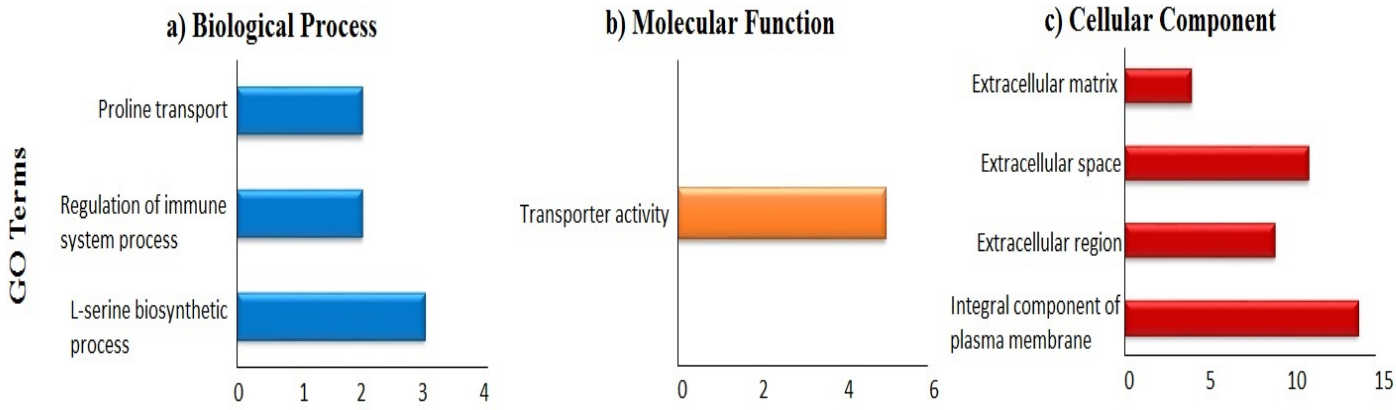

Figure 5 Gene Ontology enrichment analysis of the up-regulated genes in the magnum of laying hens. a) Biological Process, b) Molecular Function, c) Cellular Component.

3.1.3 Significant canonical pathways in magnum

After our input of the DEGs to the ingenuity pathway analysis (IPA), 417 molecules were recognized in its database that belonged to 34 significant canonical pathways (Table 9). Cell cycle control of chromosomal replication, the role of BRCA1 in DNA damage response, mitotic roles of polo-like kinase, cell cycle: DNA damage checkpoint regulation, and role of CHK proteins in cell cycle checkpoint control were the 5 most-significant canonical pathways. Among the significant canonical pathways, 2 pathways (Cell cycle: G2/M DNA damage checkpoint regulation, and regulation of cellular mechanisms by calpain protease) were predicted to be activated while 7 pathways were predicted to be inhibited; the rest lacked sufficient literatures to be predicted. We were particularly interested in the most significant metabolic pathways such as serine biosynthesis, superpathways of serine and glycine biosynthesis I, inhibition of matrix

metalloproteases, asparagine biosynthesis I, asparagine degradation I and choline degradation I (Figure 6), because these pathways reflect the underlying protein synthesis process during 3 h p.o.

Table 9 Canonical pathways identified by Ingenuity Pathway Analysis in the magnum of laying hens.

Ingenuity Canonical Pathways	-log(P-value)
Cell Cycle Control of Chromosomal Replication	11.2
Role of BRCA1 in DNA Damage Response	7.57
Mitotic Roles of Polo-Like Kinase	6.34
Cell Cycle: G2/M DNA Damage Checkpoint Regulation	5.43
Role of CHK Proteins in Cell Cycle Checkpoint Control	5.05
Hereditary Breast Cancer Signaling	4.68
Pyrimidine Deoxyribonucleotides De Novo Biosynthesis I	4.21
Serine Biosynthesis	4.18
Estrogen-mediated S-phase Entry	4.11
DNA Double-Strand Break Repair by Homologous Recombination	3.95
ATM Signaling	3.85
Superpathways of Serine and Glycine Biosynthesis I	3.64
GADD45 Signaling	3.40
DNA damage-induced 14-3-3σ Signaling	3.40
Regulation of Cellular Mechanics by Calpain Protease	3.13
Antiproliferative Role of TOB in T Cell Signaling	2.86
Neuroprotective Role of THOP1 in Alzheimer's Disease	2.69
Salvage Pathways of Pyrimidine Ribonucleotides	2.64
Atherosclerosis Signaling	2.52
Cyclins and Cell Cycle Regulation	2.43
Dopamine-DARPP32 Feedback in cAMP Signaling	2.38
Complement System	2.28
Inhibition of Matrix Metalloproteases	2.20
Pyridoxal 5'-phosphate Salvage Pathway	2.10
Hepatic Fibrosis / Hepatic Stellate Cell Activation	2.06
Airway Pathology in Chronic Obstructive Pulmonary Disease	2.03
Breast Cancer Regulation by Stathmin1	1.79
Asparagine Biosynthesis I	1.72
Cell Cycle Regulation by BTG Family Proteins	1.54
Mismatch Repair in Eukaryotes	1.44
Asparagine Degradation I	1.42

Choline Degradation I	1.42
eNOS Signaling	1.37
Cardiac β-adrenergic Signaling	1.32

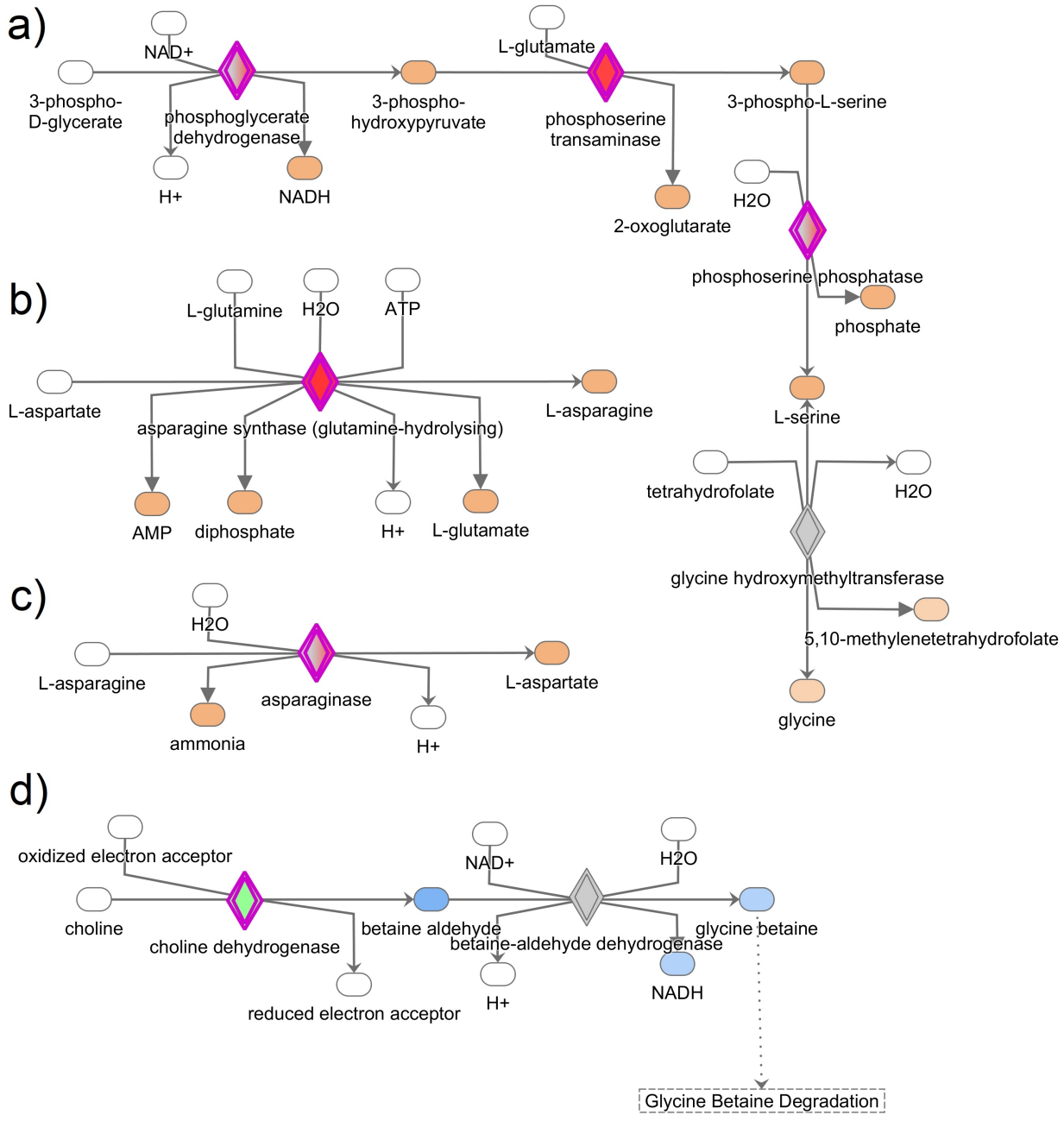

Figure 6 Canonical pathways of interest in the magnum of laying hens. a) Superpathways of serine and glycine biosynthesis I, b) Asparagine biosynthesis I, c) Asparagine degradation I, and d) Choline degradation I.

3.1.4 Gene expression profiles using qPCR in magnum

Following the identification of DEGs, the 19 most relevant up-regulated genes speculated to be related to the event of albumen synthesis were chosen for real-time PCR validation. The candidate genes were avidin (AVD), transmembrane protease serine 9 (TMPRSS9), matrix metallopeptidase 1 (MMP1), angiotensin-converting enzyme (ACE), autophagy related 10 (ATG10), avian beta-defensin 11 (AvD11), relaxin (RLN3), matrix metallopeptidase 9 (MMP9), melanotransferrin (MELTF), glutathione peroxidase 3 (GPX3), cingulin (CGN), protein C (PROC), phosphoserine phosphatase (PSPH), phosphoglycerate dehydrogenase (PHGDH), asparagine synthetase (ASNS), phophoserine aminotransferase 1 (PSAT1), matrix metallopeptidase 10 (MMP10), calpain 2 (CAPN2) and renin (REN).

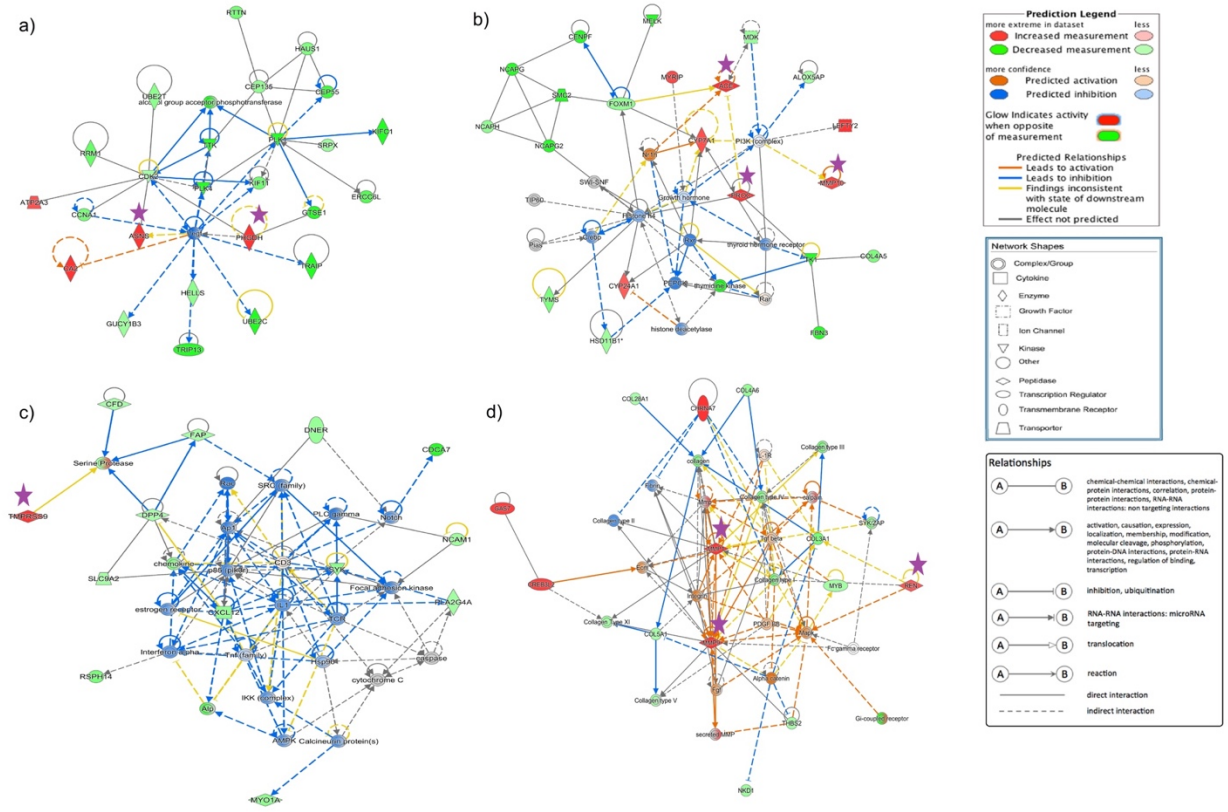

Figure 7 Gene Network showing some of the candidate genes and their interaction in the magnum of laying hens. a) PHGDH, ASNS; b) ACE, MMP10, PROC; c)TMPRSS9; and d) MMP1, MMP9, REN.

The gene network showing the interactions of some selected candidate genes using IPA network analysis is shown in Figure 7. For the qPCR analyses of the candidate genes, TBP had the most stable expression and thus, was used as a house-keeping gene. The double delta Ct ($2^{-\Delta\Delta Ct}$) method was used to calculate the relative fold change of the candidate genes after normalization with TBP. A total of ten, amongst the 19 candidate genes, showed significant changes (P-value <0.05) in expression profiles between the experimental groups (Figure 8). The mRNA expression of *CAPN2* and *PSPH* were highest only in laying hens at 15-20 h p.o. compared to either laying hens at 3 h p.o., molting, or non-laying hens. Expression of *REN*, *MMP1*, and *MMP9* mRNAs were up-regulated only in laying hens at 3 h p.o. compared to either laying hens at 15-20 h p.o., molting, or non-laying hens. Gene expression of *RLN3* was increased in laying hens, both at 3h p.o. and 15-20 h p.o. relative to molting and non-laying hens. *AVD* mRNA was highest in laying hens at 15-20 h p.o. followed by 3 h p.o., and molting hens, while least in the non-laying hens. Expression of *GPX3* mRNA was higher in laying hens at 15-20 h p.o. compared to both non-laying and laying hens at 3 h p.o. The *CGN* mRNA had increased expression in laying hens at 3 h p.o. relative to 15-20 p.o. and molting hens, while significantly higher than non-laying hens. The results of relative fold change of candidate genes obtained from RNA-Seq and qPCR were highly correlated ($R^2 = 0.94$; Figure 9).

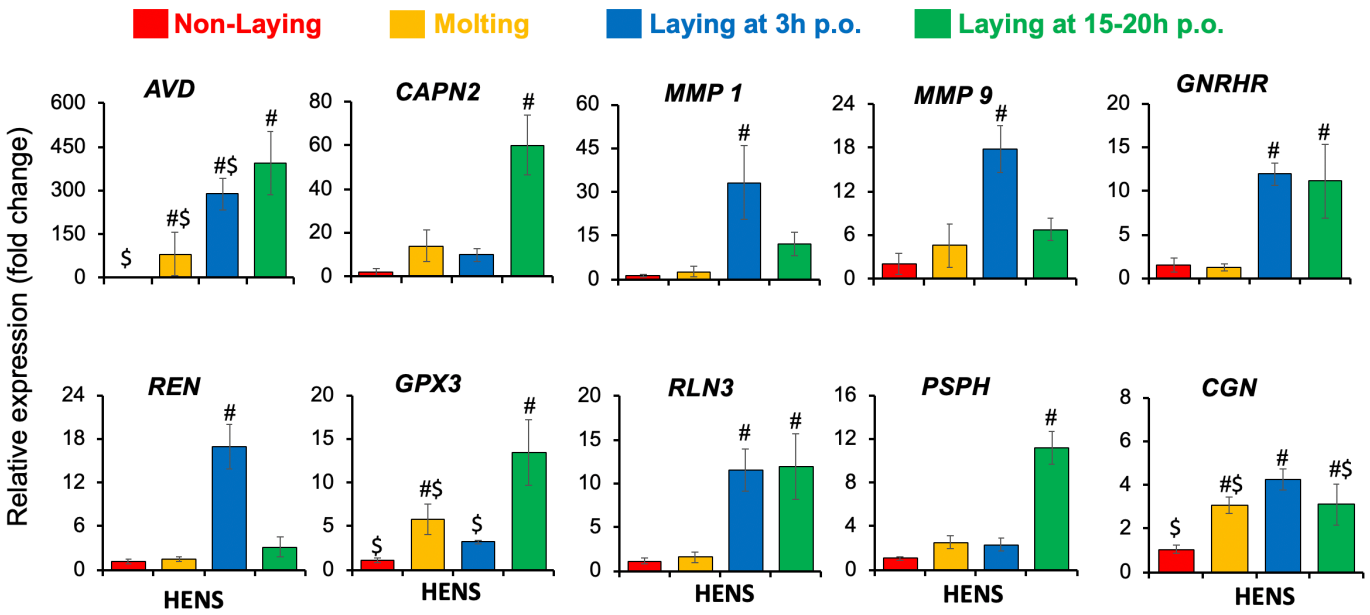

Figure 8 Validation of the gene expression in the magnum of non-laying, molting and laying hens. The fold changes were normalized with TBP gene. Data represented as the mean ± standard error. The x-axis represents a different experimental group of hens; Y-axis represents relative fold change for gene expression. # and $ denote significance at P-value < 0.05.

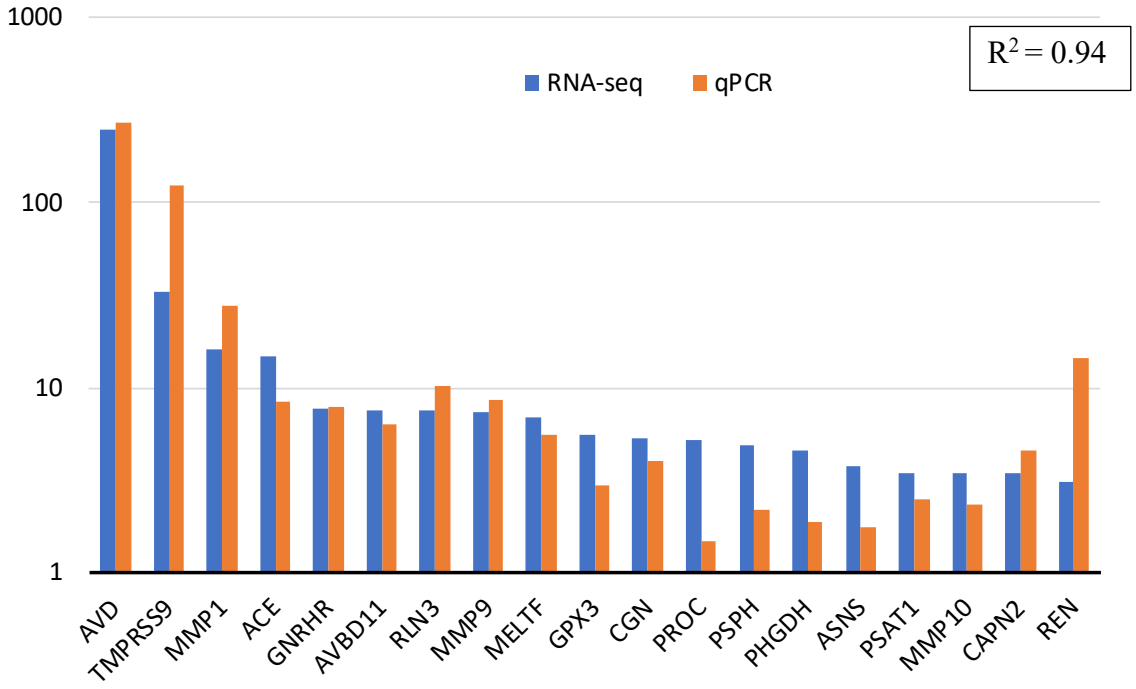

Figure 9 Correlation between the gene expression analysis in magnum by RNA-Seq and qPCR. The fold-change values are represented with a log-scale on the y-axis, whereas, the the candidate

genes are shown on the x-axis. The length of the bars represents the fold changes (layer at 3 h p.o. versus non-layers) in gene expression.

In this study, we also observed several members of the solute carrier (SLC) family of genes. The SLC genes act as exchangers, transporters, or carriers of molecules across the membranes. We performed qPCR assay for seventeen (13 up-regulated, and 4 down-regulated) members of the SLC superfamily. Results of quantitative expression showed that of the select candidate SLC genes, 9 were differentially expressed between the experimental groups of hens. SLC 26A4, SLC51B, SLC25A4, SLC1A4, SLC7A11, and SLC22A3 had the highest expression levels only in laying hens at 3 h p.o., while SLC7A17 was increased in laying hens, both at 3 h p.o. and 15-20 h p.o. compared to molting and non-laying hens. The expression of SLC16A12 and SLC 37A2 was highest in non-laying hens and the least in laying hens at 3 h p.o. (Figure 10)

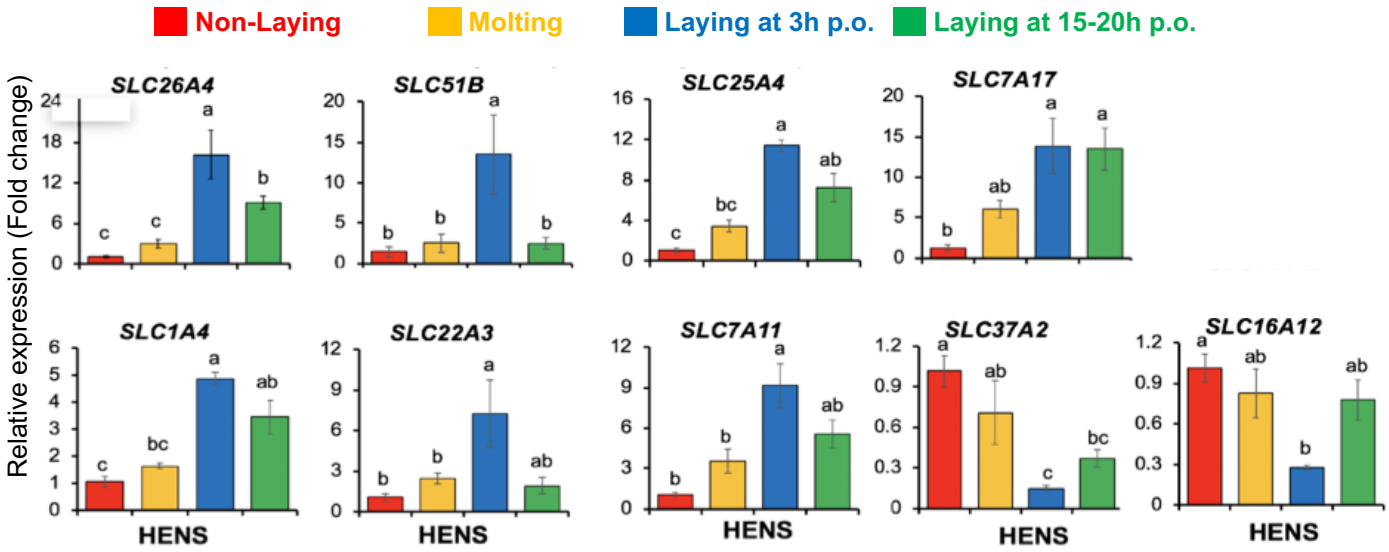

Figure 10 Gene expression profiles of select members of SLCs in the magnum of non-laying, molting and laying hens. The fold changes were normalized with TBP gene. Data represented as the mean ± standard error. X-axis represents different experimental group of hens; Y-axis represents relative fold change for gene expression. Different letterheads on the graphs denote statistical significance at P-value < 0.05.

3.2. Differentially expressed genes in the uterus

3.2.1 RNA-Seq results and identification of DEGs in the uterus

The sequencing results showed an average of 31.99 M and 28.76 M reads in layers and non-layers, respectively. After trimming and filtration, 96.39 % of input reads from layers and 96.55% input reads from non-layers were retained as excellent quality sequences (Table 10). An average of 82.96% of the retained reads from layers and 85.12% from non-layers was uniquely mapped to the genome database (Table 11). A total of 19,152 gene transcripts were annotated from Ensembl alignment which represents 50.24% of the chicken genome assembly. All the annotated and non-annotated gene transcripts were protein coding. Differential gene expression analysis showed 616 genes were differentially expressed between the layer and non-layer hens. Among the DEGs, 515 genes were officially characterized while the rest were novel transcripts without any annotation. Of the annotated DEGs, 229 genes were significantly up-regulated and 286 were significantly down-regulated in the laying hens at 15-20 h post ovulation (p.o.) when compared to the non-laying hens. The top 30 over-expressed and under-expressed genes with their relative fold change in laying hens are presented in Table 12 and Table 13, respectively. The heatmap of the 30 most up-regulated and down-regulated genes in layers with respect to non-layers is shown in Figure 11.

Table 10 Filtration summary of the RNA-Seq data in the uterus of laying hens.

	Non-layers			Layers		
Observations	Library 1	Library 2	Library3	Library 1	Library 2	Library3
Input sequences	25,772,819	30,986,225	29,524,575	30,891,969	31,025,968	34,081,293
Input bases	1,946,232,750	2,340,137,611	2,229,190,524	2,332,421,787	2,342,767,334	2,572,757,946
Input mean length	75.51	75.52	75.5	75.5	75.51	75.49
Good sequences	24,843,274	29,992,677	28,488,713	29,762,335	30,004,743	32,764,463
Good bases	1,872,511,399	2,261,115,437	2,147,276,890	2,243,159,743	2,261,820,013	2,468,939,462
Good mean length	75.37	75.39	75.37	75.37	75.38	75.35
Bad sequences	929,545	993,548	1,035,862	1,129,634	1,021,225	1,316,830
Bad bases	70,117,048	74,931,562	77,989,673	85,064,646	76,930,975	99,089,054
Bad mean length	75.43	75.42	75.29	75.3	75.33	75.25
trim_qual_left	3,727	4,153	7,148	7,173	5,935	9,812
min_len	9,427	3,763	5,985	9,726	2,884	8,466
min_qual_mean	916,391	985,632	1,022,729	1,112,735	1,012,406	1,298,552

Table 11 Mapping summary of the RNA-Seq in the uterus to the chicken genome (Galgal 5.0).

cDNA library	Reads (pre-filter)	Reads (post-filter)	Uniquely Mapped reads
Non-layer 1	25,772,819	24,843,274	20,941,092 (84.29 %)
Non-layer 2	30,986,225	29,992,677	25,975,021 (86.60 %)
Non-layer 3	29,524,575	28,488,713	24,069,813 (84.49 %)
Layer 1	30,891,969	29,762,335	25,691,008 (86.32%)
Layer 2	31,025,968	30,004,743	23,181,990 (77.26 %)
Layer 3	34,081,293	32,764,463	27,952,566 (85.31 %)

Table 12 The top 30 up-regulated genes in the uterus of laying hens.

Gene Name	Gene Description	Fold Change
OC-116	ovocleidin-116	174.00
OTOP2	otopetrin 2	15.11
TSKU	tsukushi, small leucine-rich proteoglycan	9.85
PRKG2	protein kinase, cGMP-dependent, type II	9.47
SGK1	serum/glucocorticoid regulated kinase 1	8.00
TC2N	tandem C2 domains, nuclear	6.95
NEU4	sialidase 4	6.76
GADL1	glutamate decarboxylase like 1	6.62
FGF1	fibroblast growth factor 1	5.63
LYZ	lysozyme (renal amyloidosis)	5.45
RUBCNL	RUN and cysteine-rich domain containing beclin 1 interacting protein like	5.09
GAL3ST2	galactose-3-O-sulfotransferase 2	4.92
WSCD2	WSC domain containing 2	4.76
NIPAL1	NIPA like domain containing 1	4.52
LOC427491	C2 calcium-dependent domain containing 4C-like	4.43
ROS1	ROS proto-oncogene 1, receptor tyrosine kinase	4.29
PHGDH	phosphoglycerate dehydrogenase	4.21
SLC5A9	solute carrier family 5 member 9	4.17
FST	Follistatin	4.05
FXYD2	FXYD domain containing ion transport regulator 2	4.01
MCMDC2	minichromosome maintenance domain containing 2	3.97
ALDH1L2	aldehyde dehydrogenase 1 family member L2	3.87
DIO2	deiodinase, iodothyronine type II	3.87
ATP2B2	ATPase plasma membrane Ca2+ transporting 2	3.83
NPDC1	neural proliferation, differentiation and control 1	3.69
GNRHR	gonadotropin-releasing hormone receptor	3.65
SRM	spermidine synthase	3.64
OSTN	Osteocrin	3.63
NTN3	netrin 1	3.49
OVST	ovostatin	3.45

Table 13 The top 30 down-regulated genes in the uterus of laying hens.

Gene Name	Gene Description	Fold Change
SMC2	structural maintenance of chromosomes 2	8.9721
RRM2	ribonucleotide reductase regulatory subunit M2	7.6598
CCNB3	cyclin B3	7.4819
ASPM	abnormal spindle microtubule assembly	6.9292
RACGAP1	Rac GTPase activating protein 1	6.7528
CDCA3	Cell division cycle associated 3	6.5454
CKAP2	cytoskeleton-associated protein 2	6.5290
UBE2C	ubiquitin conjugating enzyme E2 U (putative)	6.4307
BRCA1	breast cancer 1	6.3755
KNTC1	kinetochore associated 1	6.2464
CENPE	centromere protein E	6.2078
TPX2	TPX2, microtubule nucleation factor	6.0760
BUB1	BUB1 mitotic checkpoint serine/threonine kinase	6.0022
CKS1B	CDC28 protein kinase regulatory subunit 1B	5.5194
KIF11	kinesin family member 11	5.4992
NUSAP1	nucleolar and spindle associated protein 1	5.4481
ADAMTS18	ADAM metallopeptidase with thrombospondin type 1 motif 18	5.4347
DNA2	DNA replication helicase/nuclease 2	5.4128
NEK2	NIMA related kinase 2	5.4027
PLK1	polo-like kinase 1	5.2537
CENPF	centromere protein F	5.1474
CIT	citron rho-interacting serine/threonine kinase	5.1143
MELK	maternal embryonic leucine zipper kinase	4.9878
KPNA2	karyopherin subunit alpha 2	4.9424
TOP2A	topoisomerase (DNA) II alpha	4.9072
TK1	thymidine kinase 1	4.8336
ECT2	epithelial cell transforming 2	4.8087
POLQ	DNA polymerase theta	4.7944
ARHGAP19	Rho GTPase activating protein 19	4.7514
PLK4	Polo like kinase 4	4.6305

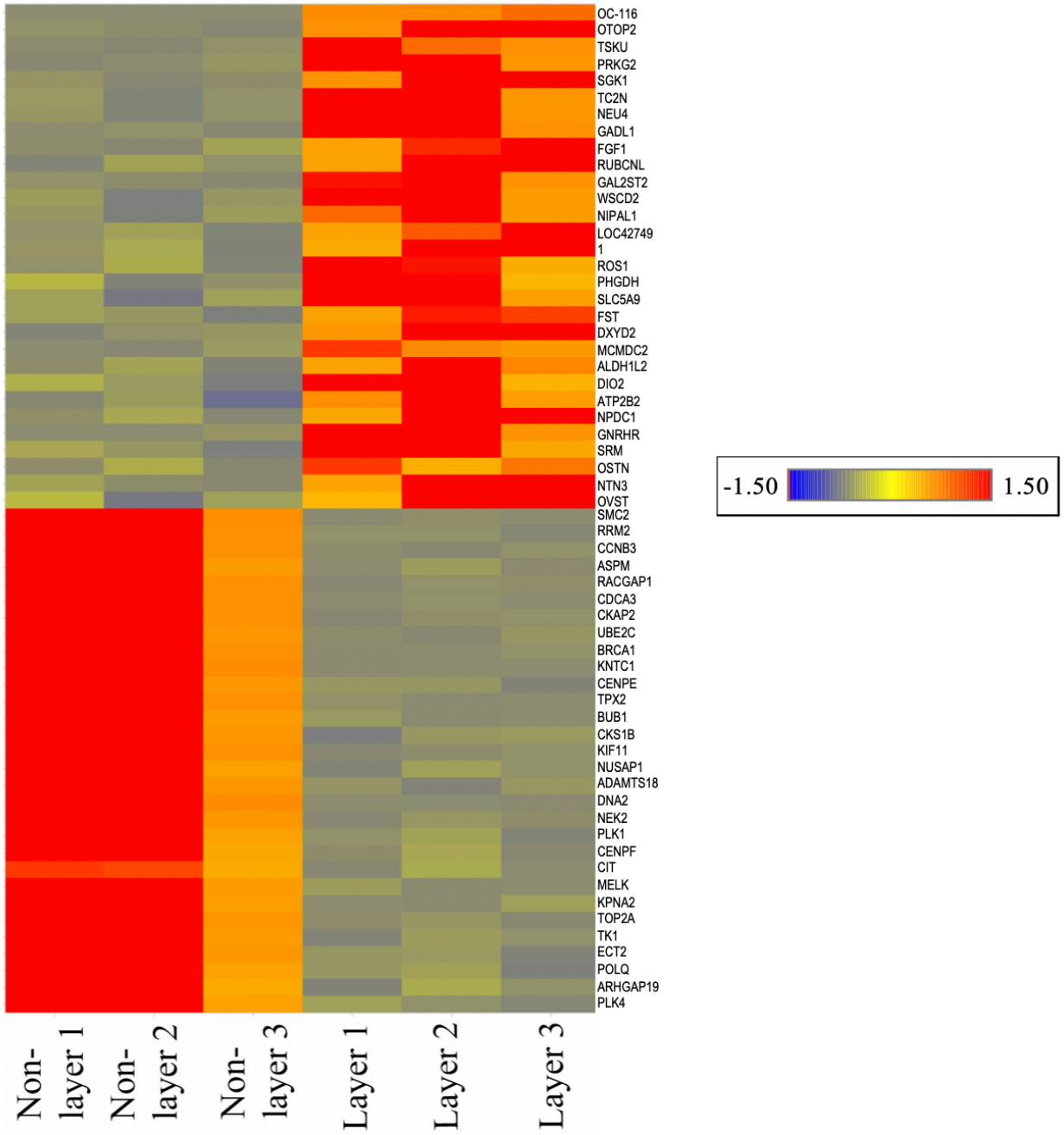

Figure 11 Heat-map of the thirty up- and thirty down-regulated genes in the uterus of laying hens. Extremes of red and purple colors indicate the highest up- and down-regulated genes, respectively, in laying and non-laying hens. The color contrasts depict the difference in the gene expression between layers and non-layers.

3.2.2 Functional annotation and pathways enrichment analysis of DEGs in the uterus

The official gene symbol of the upregulated genes was uploaded to the functional annotation tool in the DAVID system, and the chicken was selected as the reference genome. Of

the 229 genes uploaded, 193 genes were annotated. About 129 genes were enriched for biological process which included serine biosynthetic process, cellular sodium ion homeostasis, and potassium ion transport as the top three over-represented processes in the upregulated set of genes (Figure 12a). Molecular function had 3 enriched GO terms with 127 genes (Figure 12b) while cellular component contained 4 enriched GO terms with 143 genes (Figure 12c). Interestingly, the most enriched GO term for molecular function was calcium-transporting ATPase activity, while signal transducer activity and magnesium ion binding were the second and third most-enriched. Only 3 pathways were significantly enriched with pantothenate and CoA biosynthesis being the most-enriched followed by calcium signaling pathway and adrenergic signaling in cardiomyocytes (Table 14).

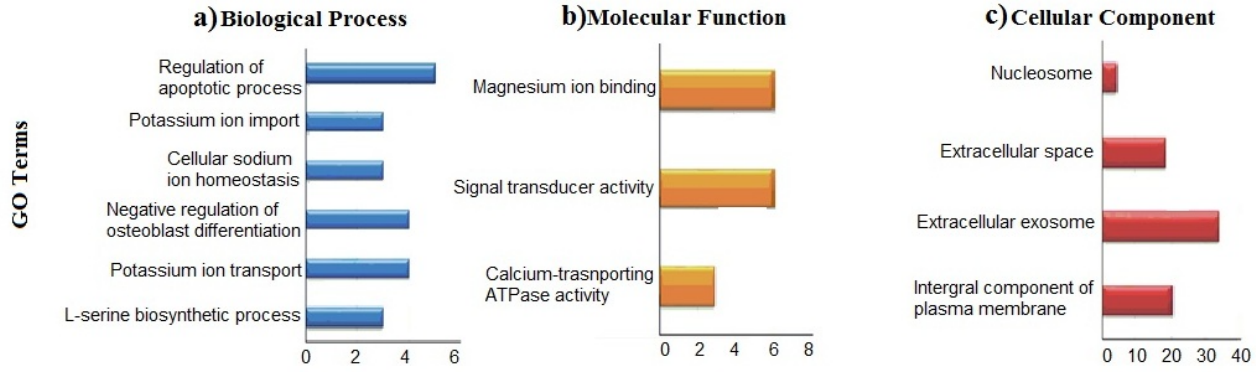

Figure 12 Gene Ontology enrichment analysis of the up-regulated genes in the uterus of laying hens. a) Biological process, b) Molecular function, and c) Cellular component.

Table 14 KEGG pathways analysis of up-regulated genes in the uterus of laying hens.

ID	Pathway terms	Fold Enrichment	Genes
gga00770	Pantothenate and CoA biosynthesis	13.6	GADL1, UPB1, VNN1
gga04261	Adrenergic signaling in cardiomyocytes	3.4	ATP2B2, ATP1B1, ADRB1, PLCB4, CREM, ATP1A1
gga04020	Calcium signaling pathway	2.96	ATP2B2, ADRB1, PLCB4, ATP2A3, ADORA2A, P2RX2, AVPR1A

3.2.3 Significant canonical pathways in the uterus

The ingenuity pathway analysis (IPA) recognized 480 molecules of the DEGs in its database and analysis showed that these molecules belonged to 43 significant canonical pathways (Table 15). Cell cycle control of chromosomal replication, cell cycle DNA damage checkpoint regulation, the role of BRCA1 in DNA damage response, mitotic roles of polo-like kinase, and estrogen-mediated S-phase entry were the 5 most-significant canonical pathways. Among the significant canonical pathways, 6 pathways (Cell cycle: G2/M DNA damage checkpoint regulation, the role of CHK proteins in cell cycle checkpoint control, cell cycle: G1/S checkpoint regulation, cAMP-mediated signaling, p53 signaling, and cardiac B-adrenergic signaling) were predicted to be activated and 9 pathways were predicted to be inhibited. The rest of the pathways were hard to predict as they had equal weight of evidence on their activation/inhibition state. Serine biosynthesis, and superpathways of serine and glycine biosynthesis I were significant with the greatest proportion of encompassed genes. We were particularly interested in calcium transport I, cAMP-mediated signaling and cardiac β-adrenergic signaling pathways because of their potential activation state, and association in ion-transport (Fig 13).

Table 15 Significant canonical pathways identified in the DEGs in the uterus of laying hens.

Ingenuity Canonical Pathways	-log(P-value)
Cell Cycle Control of Chromosomal Replication	9.68
Cell Cycle: G2/M DNA Damage Checkpoint Regulation	8.12
Role of BRCA1 in DNA Damage Response	6.73
Mitotic Roles of Polo-Like Kinase	6.70
Estrogen-mediated S-phase Entry	5.94
Cyclins and Cell Cycle Regulation	5.83
Role of CHK Proteins in Cell Cycle Checkpoint Control	5.38
GADD45 Signaling	4.33
DNA damage-induced 14-3-3σ Signaling	4.33
Hereditary Breast Cancer Signaling	4.05
Serine Biosynthesis	3.98
DNA Double-Strand Break Repair by Homologous Recombination	3.70
Antiproliferative Role of TOB in T Cell Signaling	3.63
ATM Signaling	3.49
Superpathway of Serine and Glycine Biosynthesis I	3.45
Cell Cycle: G1/S Checkpoint Regulation	3.20
Breast Cancer Regulation by Stathmin1	3.16
Calcium Transport I	2.94
Cell Cycle Regulation by BTG Family Proteins	2.90
Pancreatic Adenocarcinoma Signaling	2.85
Pyrimidine Deoxyribonucleotides De Novo Biosynthesis I	2.82
Pyridoxal 5'-phosphate Salvage Pathway	2.50
cAMP-mediated signaling	2.31
Gustation Pathway	2.13
Regulation of Cellular Mechanics by Calpain Protease	2.07
p53 Signaling	1.93
tRNA Splicing	1.90
Molecular Mechanisms of Cancer	1.82
Salvage Pathways of Pyrimidine Ribonucleotides	1.72
G-Protein Coupled Receptor Signaling	1.68
Primary Immunodeficiency Signaling	1.67
Dopamine-DARPP32 Feedback in cAMP Signaling	1.52
Oleate Biosynthesis II (Animals)	1.49
Cardiac β-adrenergic Signaling	1.44
Aryl Hydrocarbon Receptor Signaling	1.42
DNA Methylation and Transcriptional Repression Signaling	1.41
Glioma Signaling	1.38
nNOS Signaling in Skeletal Muscle Cells	1.37
Choline Degradation I	1.36
L-DOPA Degradation	1.36
Spermidine Biosynthesis I	1.36
Sulfate Activation for Sulfonation	1.36
Mismatch Repair in Eukaryotes	1.32

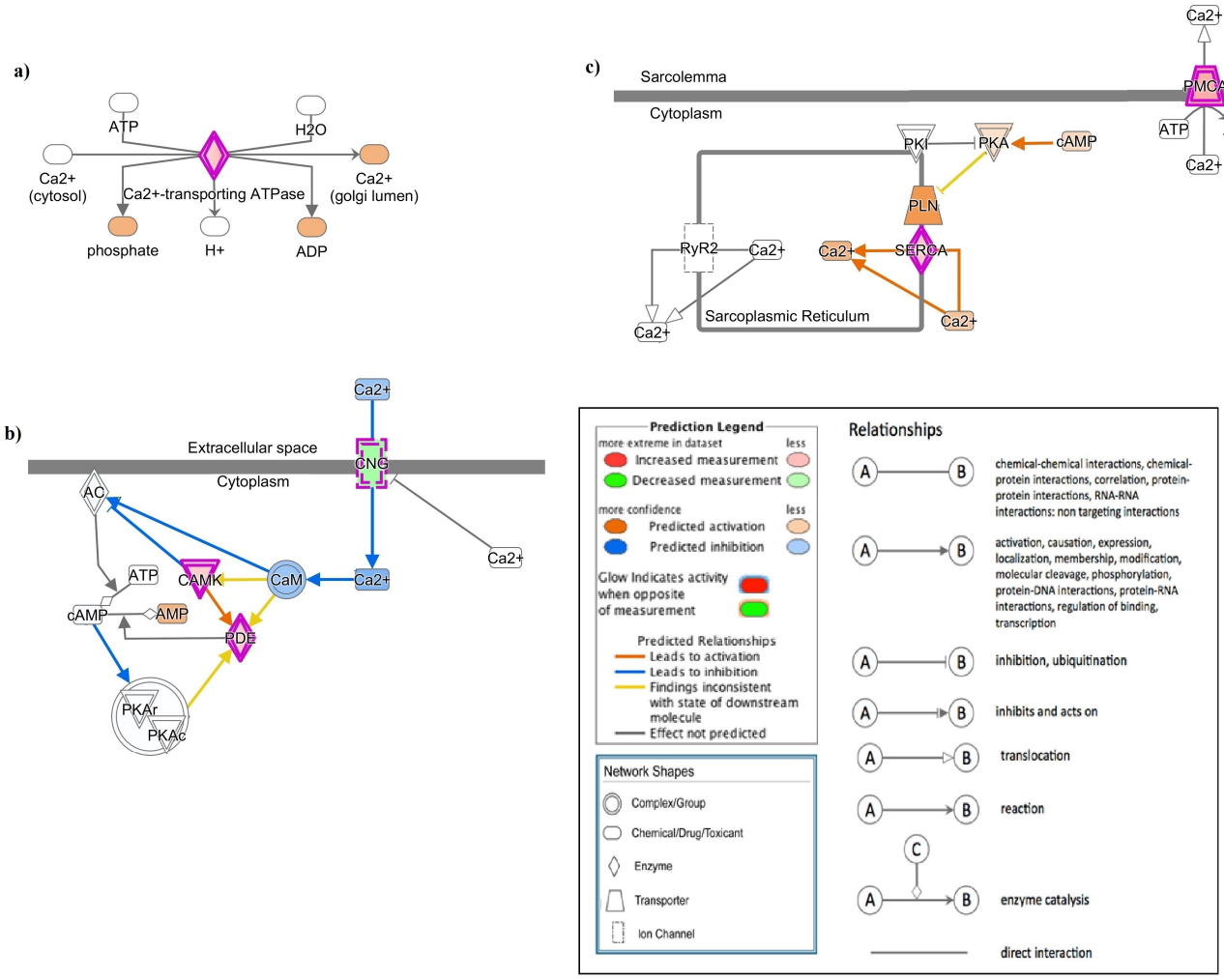

Figure 13 Results of significant canonical pathways associated with ion-transport in the uterus of laying hens and the molecules involved. a) Calcium Transport- I pathway. b) cAMP-mediated signaling pathway. c) Cardiac-β-adrenergic signaling pathway.

3.2.4 Gene expression profiles using qPCR in the uterus

The RNA-Seq data identified the differentially expressed novel genes in the uteri of laying hens. To determine whether these genes are specific to eggshell calcification, expressions of these genes were further determined in the uteri of laying hens (3h and 15-20h p.o.), molting hens and non-laying hens. Twelve candidate genes were selected for validation using qPCR: ovocleidin-116 (OC-116), otopetrin 2 (OTOP2), otopetrin 3 (OTOP3), calcitonin-related

polypeptide beta (CALCB), stanniocalcin 2 (STC2), osteocrin (OSTN), calcium/calmodulin-dependent protein kinase (CAMK1D), ATPase Na^+/K^+ transporting subunit alpha 1 (ATP1A1), ATPase sarcoplasmic/endoplasmic reticulum $Ca2^+$ transporting 3 (ATP2A3), ATPase Na^+/K^+ transporting subunit beta 1 (ATP1B1), ATPase plasma membrane $Ca2^+$ transporting 2 (ATP2B2), and ATPase secretory pathway $Ca2^+$ transporting 2 (ATP2C2). The gene network showing the interactions of the identified candidate genes using IPA network analysis is shown in Figure 14. Among the three house-keeping genes (*TATA-binding protein (TBP), GAPDH*, *B-actin*), expression of *TBP* was most stable in the uterine tissues. So, the relative fold change of the candidate genes was determined using $2^{-\Delta\Delta Ct}$ method after normalization with TBP. Of the 12 candidate genes selected for qPCR validation, five genes showed significant changes between the experimental groups. *OC-116*, *OTOP2*, *STC2* and *CALCB* had the highest expression in hens when the egg was in the uterus (15-20 h p.o.), while *ATP2C2* had the highest expression in hens when the egg was in the magnum (3 h p.o.), followed by intermediate expression intensity in hens with egg in the uterus (Figure 15). The expression of *OC-116* mRNA in the uterus was 12 thousand-fold higher and 30-fold higher in laying hens at 15-20 h p.o. compared to the non-laying and molting hens, respectively. Similarly, *OTOP2*, *STC2* and *CALCB* had 71-fold, 8-fold, and 6-fold higher expression levels in the uterus of laying hens at 15-20 h p.o. compared to the non-laying hens, respectively (Figure 15). In comparison to the molting, the uteri of laying hens at 15-20 h p.o. showed 11-fold, 3-fold, and 8-fold higher expression levels for *OTOP2*, *STC2*, and *CALCB*, respectively. The results of relative fold change for candidate genes obtained from RNA-Seq and qPCR were highly correlated (Figure 16).

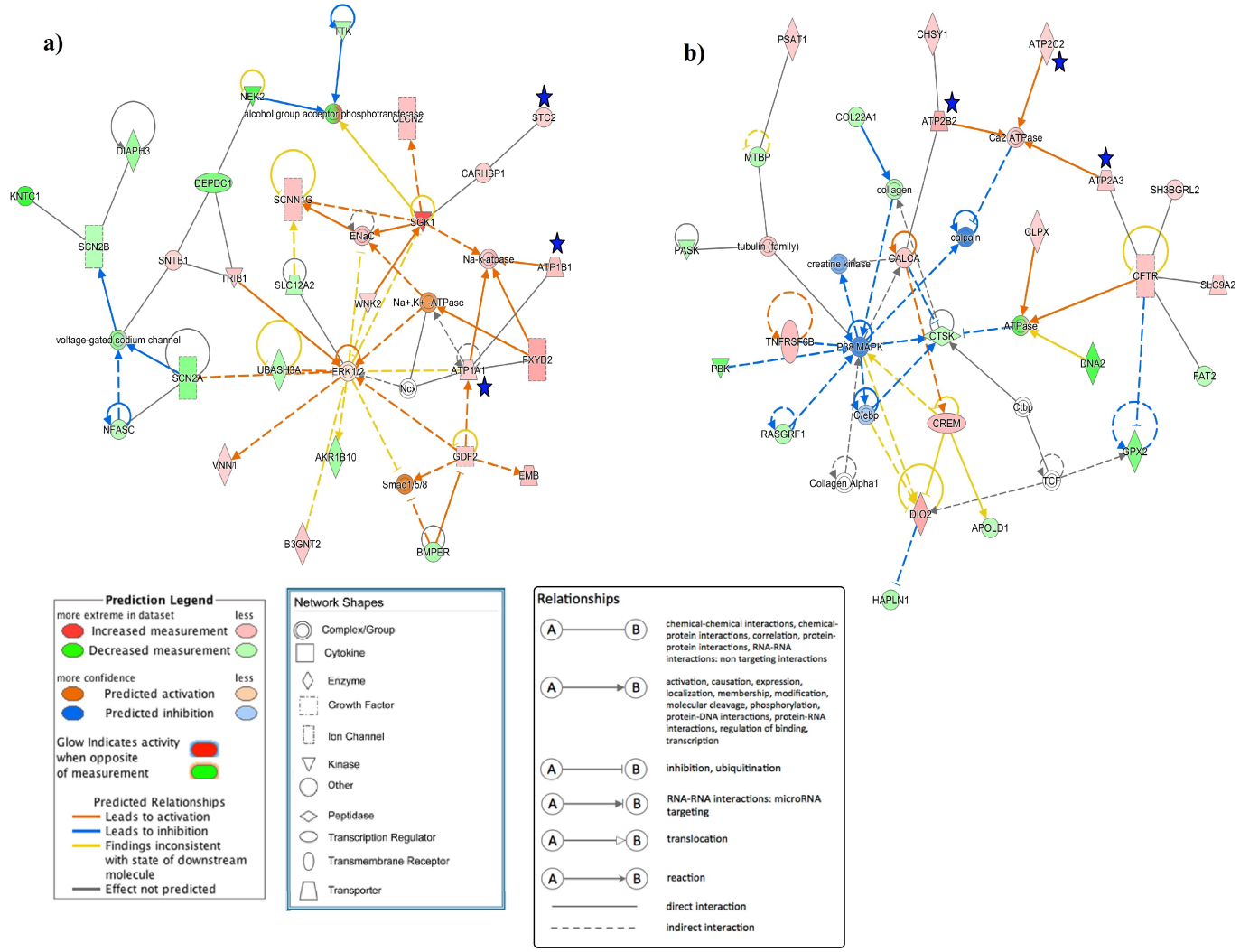

Figure 14 Gene network highlighting some of the candidate genes in the uterus and their interaction in potentially regulating the calcium-ion transport during eggshell formation. a) STC2, ATP1B1, ATP1A1; and b) ATP2B2, ATP2C2, ATP2A3.

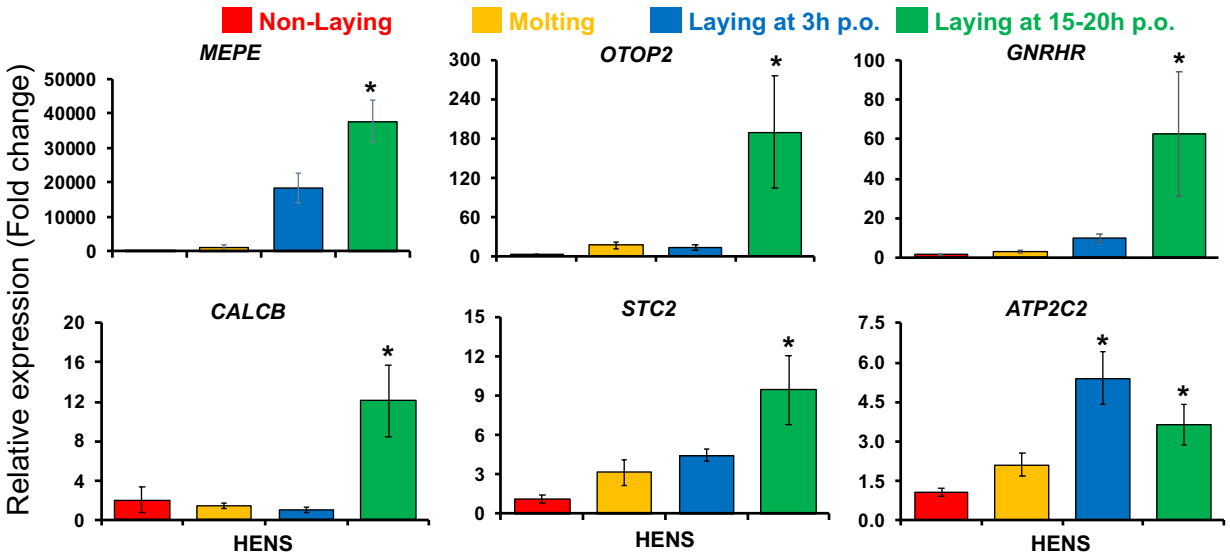

Figure 15 Validation of the gene expression in the uteri of non-laying, molting, and laying hens. The fold changes were normalized with TBP gene. Data represented as the mean ± standard error. The x-axis represents the different experimental group of hens; Y-axis represents relative fold change for gene expression. * denote significance at P-value < 0.05.

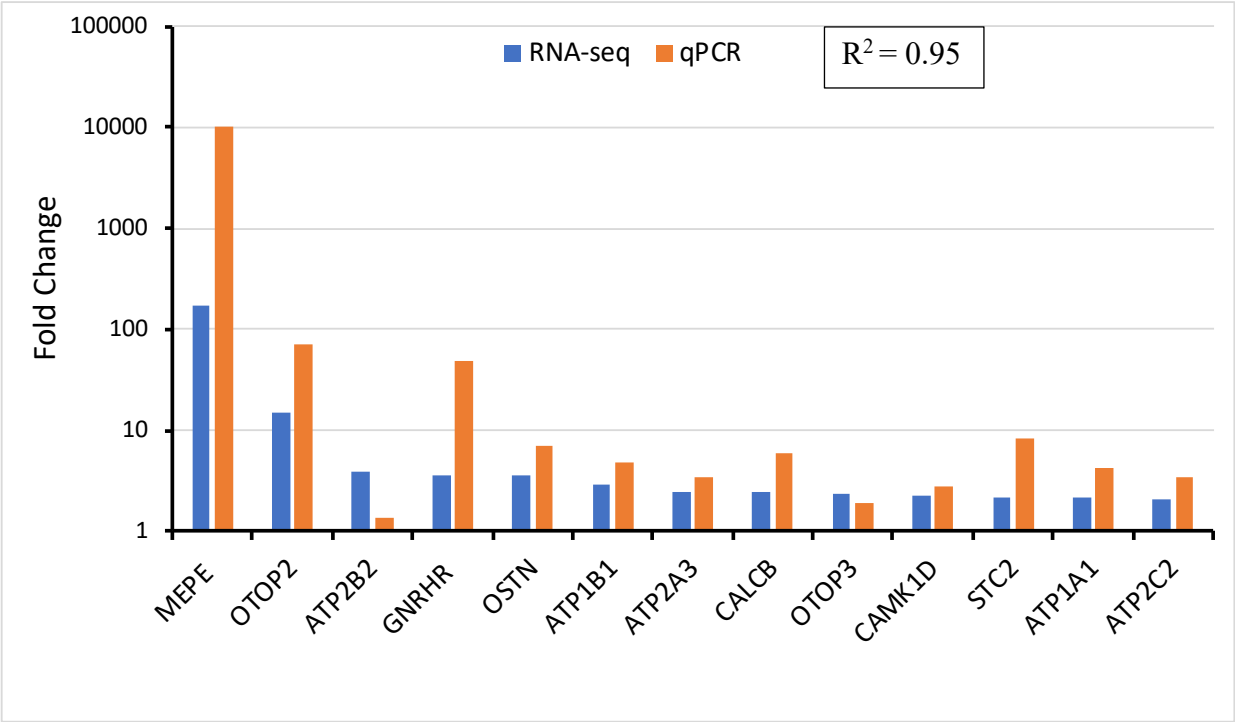

Figure 16 Correlation graph of the gene expression profiles of candidate genes in uterus measured with RNA-Seq and qPCR. The fold-change values are represented with a log-scale on the y-axis, whereas, the the candidate genes are shown on the x-axis. The length of the bars represents the fold changes (layer at 3 h p.o. versus non-layers) in gene expression.

CHAPTER 4: DISCUSSION

4.1 Candidate genes in magnum

The magnum is a highly glandular organ and secretions from the luminal and glandular epithelium contribute to the jelly portion of the egg: the albumen. The egg remains in the magnum for a short period of 1-3 hours and remaining period (4-23 hours) of the ovulation cycle in the isthmus and mostly the uterus. In the first few hours of the ovulation cycle (1-3 h p.o.), the egg is in the magnum during which the stored proteins from the magnum epithelium are secreted in the lumen (Edwards, 1976). In the later period of the ovulation cycle (4-23 h p.o.), immediately after the egg has left the magnum, the protein synthesis process begins and continues until the next egg in the cycle reaches the magnum (Edwards et al. 1976, Muramatsu et al. 1991). The albumen is a protein-rich part of the egg that imparts several critical functions such as providing nutrients and microbial defense for the growing embryo. We performed the transcriptomic analyses of the magnum in laying hens at 3 h p.o. (egg present in magnum) versus non-laying hens and compared their expression levels with laying hens at 15-20 h p.o. and molting hens using qPCR assay.

RNA-Seq data revealed a total of 540 genes to be differentially expressed between laying (at 3 h p.o.) and non-laying hens. Amongst the DEGs, several proteases (TMPRSS9, ACE, REN, MMP1, MMP9, MMP10, CAPN2, PROC) and enzymes for biosynthesis (PHGDH, PSPH, PSAT1, ASNS) were of particular interest because of their potential role in synthesis and/or secretion of egg-white proteins. Expression profiles of nine selected candidate genes, measured by qPCR assay, were different among the laying hens at 3 h and 15-20 h p.o., molting, and non-laying hens. The validated genes were involved in functions such as antimicrobial defense, matrix remodeling, albumen synthesis and/or secretion and egg transport.

4.1.1 Proteases

Proteases are enzymes having catalytic activity on proteins. There are seven different classes (based on catalytic residue) of proteases, including serine proteases and metalloproteases (Oda, 2012). Both the serine- and metalloproteases actively regulate the protein turnover of the extracellular matrix (ECM) influencing various cellular functions (Wilkins-Port et al., 2012). Our RNA-Seq and qPCR data showed that *TMPRSS9* was the second most highly expressed gene in laying hens when the egg was inside the magnum. The qPCR data showed a relatively higher expression of *TMPRSS9* in laying hens at 20 h p.o. followed by 3 h.o. compared to molting and non-laying hens, however, it was non-significant due to higher standard errors (data not shown). TMPRSS9, also known as polyserase-I, is a transmembrane type II serine protease that uniquely produces three other proteases, including 2 active ones (Cal et al., 2003). TMPRSS9 facilitates the formation of urokinase plasminogen activator that converts plasminogen to plasmin responsible for degradation of ECM components and activation of procollagenases (Fontanil et al., 2014). Higher expression of *TMPRSS9* in laying hens suggests that it potentially participates in degradation of the ECM to release the stored proteins making them available for receptor binding and signaling action, as proposed by Ten Dijke et al.(2007). This process is indeed relevant in laying hens both when the egg is present in the magnum or the shell gland for keeping up with the massive amount of protein synthesis and secretion.

Matrix metalloproteases (MMPs) are the major regulators of ECM remodeling. There are currently 25 members in the MMPs family in vertebrates, including MMP-1, -9, and -10 which are secreted proteins involved in wide range of physiological activities such as cellular migration, neoangiogenesis, and inflammation (Page-McCaw et al., 2007). In our study, expression of *MMP-1* and *MMP-9* were upregulated in laying hens with the presence of egg in the magnum, compared to rest of the physiological stages of experimental hens. MMP1, also

known as collagenase, is capable of degrading most highly abundance ECM; collagen in several tissues including chicken ovary (Zhu et al., 2014). MMP-9 (gelatinase) is known to degrade gelatin matrix (Sternlicht and Werb, 2001), provokes angiogenesis (Bergers et al., 2000), and also regulates the laying process in hen (Lesniak-Walentyn and Hrabia, 2016a). In the current study, expression of *MMP-9* was upregulated in the magnum at 3h p.o. (both RNAseq and qPCR) suggesting it's potential role in albumen formation. MMP10 is known to break down the several types of collagen-related connective tissues (Muller et al., 1998). RNA-Seq data in this study showed that *MMP10* mRNA is expressed more by 3.4-fold in the magnum of laying hens than that in non-laying hens. This is the first study to report *MMP10* mRNA expression in the chicken oviduct. However, study of cervical tumors in humans has confirmed association of MMP10 in angiogenesis of cervix (Zhang et al., 2014). The vascular endothelial growth factor (VEGF) which plays a critical role in endothelial cell migration, tube formation and microvessel sprouting stimulates the mRNA expression of MMP10 through the protein kinase D1-histone deacetylases 7 (HDAC7) dependent pathway (Ha et al., 2008). This association can be used to infer the functional role of MMP10 in the magnum. For albumen formation, several proteins need to be synthesized and transported into the lumen for deposition around the egg yolk. The required proteins are synthesized in the tubular gland cells of magnum but require the rapid transport of amino acids from the blood circulation (Edwards et al., 1976). We believe that, the MMPs over-expression in this study demonstrates their associated role in tissue remodeling and formation of new vasculatures to support the expeditious convey of precursor molecules for biosynthesis of albumen.

Calpains, on the other hand, are ubiquitous intracellular cysteine proteases having very low specificity for recognition of amino acid sequence. Calpains have wide range of functions in various tissues including membrane repair, cell adhesion and motility, cell death, protein

cleavage, and activation (Ono and Sorimachi, 2012). Our study reports an increased expression of CAPN2 in laying hens during albumen secretion period, while yet several folds higher expression during the albumen secretion period compared to either molting or non-laying hens. We, therefore, posit that CAPN2 is responsible for maturation and activation of the synthesized egg-white proteins.

4.1.2 Antimicrobials

Antimicrobial agents are crucial for the livability of the hen's embryo. The albumen holds the yolk (with ovum) in the center of the egg, without any contact to the eggshell. It acts as a thick protective layer consisting of several antibacterial proteins. One such established protein is avidin, and interestingly in our study, the *AVD* was the most overly expressed in laying hens. Avidin has a very high affinity for biotin-required for bacterial growth and proliferation and thus, prevents the invasion by microbial pathogens (Krkavcova et al., 2018). Other newly discovered and widely studied chicken antimicrobial protein is avian beta-defensins (AvBDs). AvBD11 is one among the 14 members of the AvBDs whose mRNA expression was increased in the magnum of laying hens in our study. Similar observation of *AvBD11* was reported by Lim et al. (2013). Additional studies have shown the presence of AvBD11 in vitelline membrane of egg, eggshell membrane, and eggshells (Mageed et al., 2009; Herve-Gripenet et al., 2010). Above findings suggest that the AvBD11 is an important molecule for innate immunity in hens. AvBD11 incorporation in the albumen protects the developing embryo and might increase hatchability of the eggs.

4.1.3 Molecules involved in the biosynthesis

Several enzymes such as PHDGH, PSPH, PSAT1, ASNS, ASPG, GALNT6, PDE3A, and PHYKPL were increased in laying hens as shown by our RNA-Seq data. GO enrichment analysis additionally revealed that PHDGH, PSPH, PSAT1, and ASNS were involved in amino-

acid biosynthesis. The biosynthesis of L-serine from 3-phosphoglyceraldehyde is mediated by three enzymes PHGDH, PSAT1, and PSPH at each successive step, respectively (Chen et al., 2013). Interestingly, the mRNA of *PHGDH* had increased expression in laying at 15-20 h p.o. (during the albumen synthesis period), while *PSAT1* and *PSPH* mRNAs were also relatively higher in those hens. This finding provides strong evidence indicating the biosynthesis of serine in magnum which may be required for synthesis of egg white proteins. Report by Li et al. (2016) suggests an additional role of serine biosynthesis enzymes (PHGDH, PSAT1, and PSPH) in protection from reactive oxygen species (ROS) by providing the substrate-serine, for glutathione synthesis. Indeed, the antioxidative function of serine biosynthesis enzymes in magnum is plausible since, cells of the magnum are involved in the production of the huge amount of proteins, and concurrently ROS as bi-products.

4.1.4 Antioxidants

Glutathione peroxidase (GPX) is a well-known enzyme capable of protecting the cells and tissues from ROS such as hydrogen peroxidases and another lipid hydroperoxides. GPX3 is an isoform of the enzyme GPX class which is localized in plasma and extracellular spaces (Takahashi and Cohen, 1986). In our study, RNA-Seq showed increased expression of *GPX3* in the magnum of laying versus non-laying hens, while qPCR confirmation also showed an upregulated expression in laying hens during albumen synthesis period and molting hens in relation to non-laying hens. The findings are indeed concurrent with the underlying physiological activities in laying and molting hens. In the magnum of laying hens, rapid protein synthesis occurs at 4 – 23 h p.o. meaning that cells of magnum have increased metabolism. As a result, simultaneous with protein synthesis, there is the release of ROS and other free radicals. So, the increased *GPX3* expression in magnum is indicative of the protective response against oxidative damage. In molting hens, the oviduct is undergoing rejuvenation, and there is huge tissue

turnover with the production of free radicals (Sundaresan et al., 2007). Thus, the higher levels of GPX3 expression is associated with its antioxidative function in magnum. In addition, several other genes differentially expressed in laying hens, such as *urotensin 2* and *spermine oxidase* functioning in the production of ROS and hydrogen peroxide (Yu et al., 2015; Chaturvedi et al., 2012), respectively, support the fact that oxidative stress is evident in the magnum.

4.1.5 Genes involved in albumen secretion and/or oviductal transport of egg

Relaxin hormone produced from the ovary and placenta in mammals helps ease the parturition process by relaxing the ligaments and dilating the cervix. The relaxin-like family peptide has seven peptides, including relaxin-3 which belong to the insulin superfamily. However, the phylogenetic study showed that the chicken genome has lost all the relaxin family peptides, but relaxin-3 having high homology to the human analog (Wilkinson et al., 2005). Relaxin-like peptide is produced in granulosa cells of the post-ovulatory follicles, localized in the uterus of laying hens, and influences the oviduct and uterus to aid in oviposition (Brackett et al., 1997). Also, loss in functionality of this avian relaxin, through surgical removal of the granulosa cells of post-ovulatory follicles, results in a drastic delay in oviposition time (Wood-gush and Gilbert, 1964; Gilbert et al., 1978). Studies of Brackett (1997) and Wilkinson (2005) suggest that the hormonal action of relaxin-3 from ovaries help in egg-laying. In addition, our study detected a significant expression of *RLN3* mRNA in the magnum of laying hens both during albumen synthesis and secretion period. RLN3 expression in the magnum is a novel finding suggesting the synthesis of relaxin protein in the magnum, as well. So, we hypothesized that its over-expression at 3 h p.o. in the oviduct may be related to the mechanical distention of the magnum to ease the passage of the developing egg and/or secretion of the stored egg-white proteins. Since the mechanical pressure on the walls of the magnum provokes the secretion of the

synthesized albumen proteins (Sturkie and Mueller, 1976), *RLN3* potentially is one of the markers of mechanical stimulus for the secretion of albumen from goblet cells of magnum. The renin-angiotensin system (RAS), besides its well-known endocrine role in maintaining extracellular fluid in the body, also regulates the ovarian growth dynamics (Yoshimura, 1997). Renin found in ovarian theca cells (Fernandez et al., 1985; Do et al., 1988), and angiotensin-converting enzyme (ACE) localized in the granulosa cells and blood vessels of the ovary (Speth and Husain, 1988) are the principle components of the RAS system. Apart from the endocrine function of RAS, the localized action of RAS in the ovary is towards follicular development and ovulation (Goncalves et al., 2012). In our study, *REN* mRNA had significantly increased expressions in the magnum tissues of laying hens during albumen secretion period as compared to moting and non-laying hens. The *ACE* mRNA was also higher in laying hens relative to non-laying hens. There are few reports on the activity of RAS in the uterus of humans (Li and Ahmed, 1997), rats (Pawlikowski et al., 1999), rabbit (Eskildsen, 1972) and quail (Verma and Panda, 1992). However, to our knowledge, there is no study of RAS in the chicken oviduct. Verma and Panda (1992) reported the expression of ACE in the oviduct of both immature and mature (with exogenous estrogen) quails with the highest expression in magnum among the other oviductal parts. REN and ACE, chief molecules of the RAS, are predominantly found in the glandular epithelium of the human uterus where the RAS system had different roles during the menstrual cycle (Li and Ahmed, 1997). Based on our findings and the unique role of magnum in egg formation, we support the hypothesis proposed by Verma and Panda (1992) that the RAS system may control the blood supply to the magnum by altering the vascular smooth muscle tone (through bradykinin), and formation of new blood vessels (Khakoo et al., 2008). The RAS system, specifically in the magnum, might aid in relaxing the magnum to retain the egg for suffice period, allowing optimum deposition of albumen.

The above studies in association with the findings of this study suggest that the expression of *REN* and *ACE* in the magnum of laying hens is a strong evidence that RAS system is also involved in the oviductal transport of egg in chicken.

4.1.6 Solute carriers

The solute carrier is a superfamily, the members of which are membrane transport proteins. Most of the proteins are located on the plasma membrane which carry, exchange, or transport solutes across the membranes. RNA-Seq and qPCR assays confirmed the differential expression of several members of the SLC family in the magnum of laying and non-laying hens. The up-regulated expression of *SLC26A4* (anion exchanger), *SLC51B* (organic solute transporter), *SLC25A4* (mitochondrial carrier), *SLC1A4* (neutral amino acid transporter), *SLC7A11* (anionic amino acid transporter), and *SLC22A3* (organic cation transporter) in the magnum at 3 h p.o. suggests that indeed, these SLCs are directly involved in the transport of precursors in and across the magnum epithelium for albumen synthesis. SLC7A17 had increased expression in laying hens at times when the egg was in magnum and in the uterus, which indicates a sustained expression of SLC7A17 (neutral amino acid transporter) in laying hens during the overall period of egg formation. This is quite plausible for the reason that albumen synthesis in the magnum is a continuous process (Edwards et al., 1976).

4.1.7 Regulator of cellular tight junctions

Cingulin is a protein localized at the tight junction of epithelial and endothelial cells, first discovered in the chicken intestine, and involved in creating a barrier for molecular transport across cells (Citi et al., 1988). RNA sequencing data in our study showed significantly higher expression of *CGN* in laying hens, and qPCR results further confirmed that laying and molting hens have similar levels of *CGN* expression that is significantly greater compared to non-laying hens. Cingulin is involved in the organization of the tight junctions, but simultaneously, it

inhibits RhoA (Ras homolog gene family member A) activation and suppresses epithelial cell proliferation and gene expression (Balda et al., 2009). However, it is also implicated that CGN regulates cell growth and morphology and creates a single layer of small, tightly packed cells (Guillemot and Citi, 2006). To the best of the available literatures on CGN function, we believe that *CGN* is involved in the cellular organization and integrity of the magnum epithelium in laying hens regulating molecular transport across the epithelial barrier.

4.2 Candidate genes in the uterus

Eggshell calcification in the uterus of laying hen involves several cellular and molecular processes in the secretion, transport, and biomineralization of calcium carbonate around the egg. In this study, we performed gene expression profiling using RNA-Seq of uterine samples from laying and non-laying hens. DESeq analysis between layers' and non-layers' uteri revealed several novel genes and biological pathways that potentially regulates the calcium transport and consequently the eggshell formation. Based on the enriched pathways and molecular functions, and their expression levels, twelve genes were identified as potential regulator of calcium-homeostasis during eggshell mineralization. Further, these novel genes were validated in the uteri of laying (3 h p.o. and 15-20 h p.o.), molting and non-laying hens using qPCR. Among the upregulated genes, *OC-116*, *OTOP2*, *STC2*, *CALCB*, and *ATP2C2* were significantly higher in the uteri of laying hens during the eggshell formation (15 to 20 h p.o.).

Ion-transporting genes/proteins have a significant role in supplying the required amount of calcium and other ions for eggshell mineralization. Previous transcriptomic studies (Dunn et al., 2009b; Jonchere et al., 2010, 2012; Jeong et al., 2012; Brionne et al., 2014; Zhang et al., 2015) in the chicken oviduct have reported that calbindin, ATPases, sodium calcium exchangers, and solute carriers are actively involved in the supply of ions and minerals for eggshell

biomineralization. Some well-known genes regulating eggshell mineralization such as calbindin, secreted phosphoprotein, and regulator of calcineurin were detected in our RNA-Seq data, and we confirmed their differential expression by qPCR (data not shown).

The OC-116 also known as MEPE (matrix extracellular phosphoglycoprotein) is an anti-remodeling matrix protein and plays a role in bone- and eggshell- mineralization (Hincke et al., 1999; David et al., 2009; Jonchere et al., 2012). The OC-116 is the principle protein of the eggshell matrix which is associated with the microvesicular cavities of the eggshell and regulates the calcite crystals organization within the eggshell. It determines the elasticity, shell thickness, and eventually the shape of the egg (Nys, 2004; Dunn et al., 2009a; Hincke et al., 2012; Zhang et al., 2015). Both microarray and proteomic studies have confirmed that the expression of OC-116 is dominant at the active calcification stage of mineralization (Dunn et al., 2009b; Jonchere et al., 2010; Brionne et al., 2014; Zhang et al., 2015; Marie et al., 2015; Miksik et al., 2007). We also observed a very high expression of *OC-116* in the uteri (174-fold by RNA-Seq and 12,000-fold by qPCR) during the active eggshell calcification. Similar to our findings, expression of *OC-116* has also been reported by Brionne et al. (2014), where relative expression was determined in laying hens and expelled hens (same as non-laying) that mimics the experimental model of this study confirming the role of *OC-116* in eggshell biomineralization.

Calbindin is an intracellular calcium ion transporter and maintains a low concentration of Ca^{2+} in the uterus (Jonchere et al., 2012). In our study, we identified some previously unpredicted otopetrin genes that may participate in the trans-epithelial transport of Ca^{2+} across the uterine plasma membrane into the cytoplasm. The otopetrin gene family has three members; OTOP1, OTOP2, and OTOP3. Though the function of OTOP2 and OTOP3 remains unknown, *Otop1* is postulated to maintain the high concentration of cytosolic calcium in the supporting cells of inner ear during otoconia mineralization in mice (Kim et al., 2010). OTOP genes do not

necessarily have the same biochemical function as *Otop1* (Hurle et al., 2011), however, based on their highly conserved nature between vertebrates and a validated up-streamed expression in laying hens during egg calcification, we hypothesized that OTOP2 has a similar role of regulating intracellular calcium in the uterus for eggshell calcification. The ion-transporting function of otopetrin is dependent on changes in the cellular pH (Tu et al., 2018) which is very evident in uterine cells during mineralization process.

Stanniocalcin 2 (STC2) and calcitonin-related polypeptide B (CALCB), also identified for the first time in the uterus, were significantly increased in the laying hens at 15-20h p.o. STC2 is distributed in a wide variety of tissues and has several functions including osteoblast differentiation (Kim et al. 2015; Zhou et al., 2016). It is expressed in chicken joints, and its expression is directly related to bone mineralization (Mittapalli et al., 2006; Zhou et al., 2016). STC2 acts via activation of extracellular signal-regulated kinase ½ (ERK1/2) pathway (Zhou et el., 2016). The ERK5 pathway, observed in our RNA-Seq analysis, has a similar function to the ERK1/2 pathway (Nishimoto and Nishida, 2006). CALCB is a member of the calcitonin-related polypeptide family primarily known to regulate cellular calcium homeostasis. It was overexpressed only in the laying hens at 15- to 20-h p.o. compared to non-layers, molters, and layers at 3h p.o, suggesting its primary role in eggshell mineralization in hen uteri. CALCB in the uterine epithelium may act through activation of IP3 secondary messenger to cause the intracellular release of free Ca^{2+} ions from the endoplasmic reticulum reserve (Russell et al., 2014). Our findings and earlier reports are indicative that STC2 and CALCB are also the novel regulators of mineralization in the eggshell.

The present study also reports the expression of several members of the ATPase family genes in the uterus of laying hens. ATPases are groups of enzymes that help to transport solutes across the cell membrane against their concentration gradient. Three types of ATPases are

present in mammals and birds; plasma membrane Ca^{2+} ATPase (PMCAs), secretory pathway Ca^{2+} ATPase (SPCAs), sarcoplasmic reticulum Ca^{2+} ATPase (SERCAs). ATP2B2 is a member of the PMCAs and catalyzes the export of calcium ions in the extracellular space (Carafoli et al. 2002). Based on IPA analysis and canonical pathways, ATP2B2 participates in the calcium signaling pathway which is predicted to be activated during eggshell mineralization. ATP2C2 is a member of the SPCA family of genes. IPA analysis showed that ATP2C2 is a molecule in the significant Calcium Transporting I canonical pathway. It regulates the influx of Ca^{2+} into the cell and the consecutive transport of the cytosolic calcium ions into the Golgi lumen (Smaardijk et al., 2017). Expression of ATP2C2 in cancer cells is followed by an increase in luminal Ca^{2+} and initiation of microcalcifications (Dang et al., 2017). Based on the functional role of ATP2C2 in different tissues, we speculated that it might participate in eggshell mineralization. We also found ATP2A3 significantly up-regulated in uteri of laying hens. ATP2A3, the member of SERCAs, is an intracellular calcium pump and translocates the cytosolic Ca^{2+} ions to the sarcoplasmic reticulum lumen. Its' up-regulated status in the uterus suggests that ATP2A3 helps in the act of oviposition.

The top 15 significant canonical pathways belonged to cell cycle, proliferation, and biosynthesis which is reflective of the cellular changes and biological activity occurring in the uterus. However, the focus of this study was on the canonical pathways those are directly related to ion-transport. The DEGs belonged to three significant canonical pathways of interest; 1) Calcium Transport-I, 2) cAMP-mediated signaling, and 3) Cardiac β-adrenergic signaling. Calcium Transport-I pathway constituted of ATP2A3, ATP2B2, and ATP2C2 and involved in the transport of Ca^{2+} ions from the cytosol into the golgi lumen (Figure 13a). Calcium/calmodulin-dependent protein kinase ID (CAMK1D) was one of the molecules that act through the cAMP-mediated signaling pathway. Cyclic nucleotide gated channel alpha 4

(CNGA4), a down-regulated molecule in layers observed in our RNA-Seq, was also involved in the cAMP-mediated signaling. CNGA4 has Ca^{2+} mediated negative feedback on the CAMK1D activation (Munger et al., 2001; Figure 13b). The SERCAs act via the Cardiac β-adrenergic signaling pathways (Figure 13c). The ATPases governed the three canonical pathways associated with calcium-ion remodeling. However, the IPA canonical pathways analysis failed to incorporate the other novel molecules such as OTOP2, STC2, and CALCB, possibly, due to the insufficiency of the regulatory and mechanistic information of such novel genes in the uterus.

The eggshell biomineralization in the hen uteri is a complex process involving the interaction of organic matrix and inorganic solutes to form a hard-calcified membrane that protects the inner content of the egg. With the development of transcriptomic methods, new genes associated with the biological process are being discovered. Using Next Generation Sequencing analysis of uteri, we report the involvement of five novel genes in eggshell mineralization. We further confirmed that *Stanniocalcin 2*, *Otopetrin 2*, *Calcitonin-related polypeptide B* and *ATPase secretory pathway $Ca2^{+}$ transporting 2* are the novel genes in the laying hen uterus that are associated with calcium-ion transport and thus, are essential molecules in the eggshell formation. Also, the canonical pathways such as calcium transport I, cAMP-mediated signaling, and cardiac β-adrenergic signaling were identified to be associated with ion-transport during eggshell formation.

4.3 Conclusion and future directions

We were able to answer our research questions from the results of the RNA sequencing and real-time PCR assay. The magnum and the uterus are the specific sites for albumen and eggshell formation, respectively. Both the RNA-Seq and qPCR confirmed the differential

expression of several pre-discovered and some novel genes and their biological pathways relevant to egg formation in the oviduct of laying hens.

The series of events that occurs in the magnum contributing the albumen include transport of precursor molecules, synthesis of proteins, and secretion of the synthesized proteins to be deposited around the egg yolk. This study finds some important molecules that elucidate the cascade of events associated with the albumen deposition in the magnum. The proteases such as, CAPN2, TMPRSS9, MMP1, and MMP9 are involved in protein maturation and activation, ECM degradation and angiogenesis for the transport of several precursor molecules from the blood circulation so that the magnum epithelium can utilize them for the synthesis of egg-white proteins. The enzymes PHGDH, PSPH, and PSAT1 directly participate in the synthesis of amino-acids that are basic units of the complex albumen protein. Relaxin-3, and renin-angiotensin system (REN and ACE) regulates the transport of egg through the oviduct controlling how long the egg stays in the magnum. Also, they ease the secretion of albumen from the granular cells for deposition around the egg. Besides, the fundamental albumen proteins, some other molecules which have protective function to the egg such as, AVD, AvBD11, and GPX3 are incorporated in the egg albumen (Figure 17).

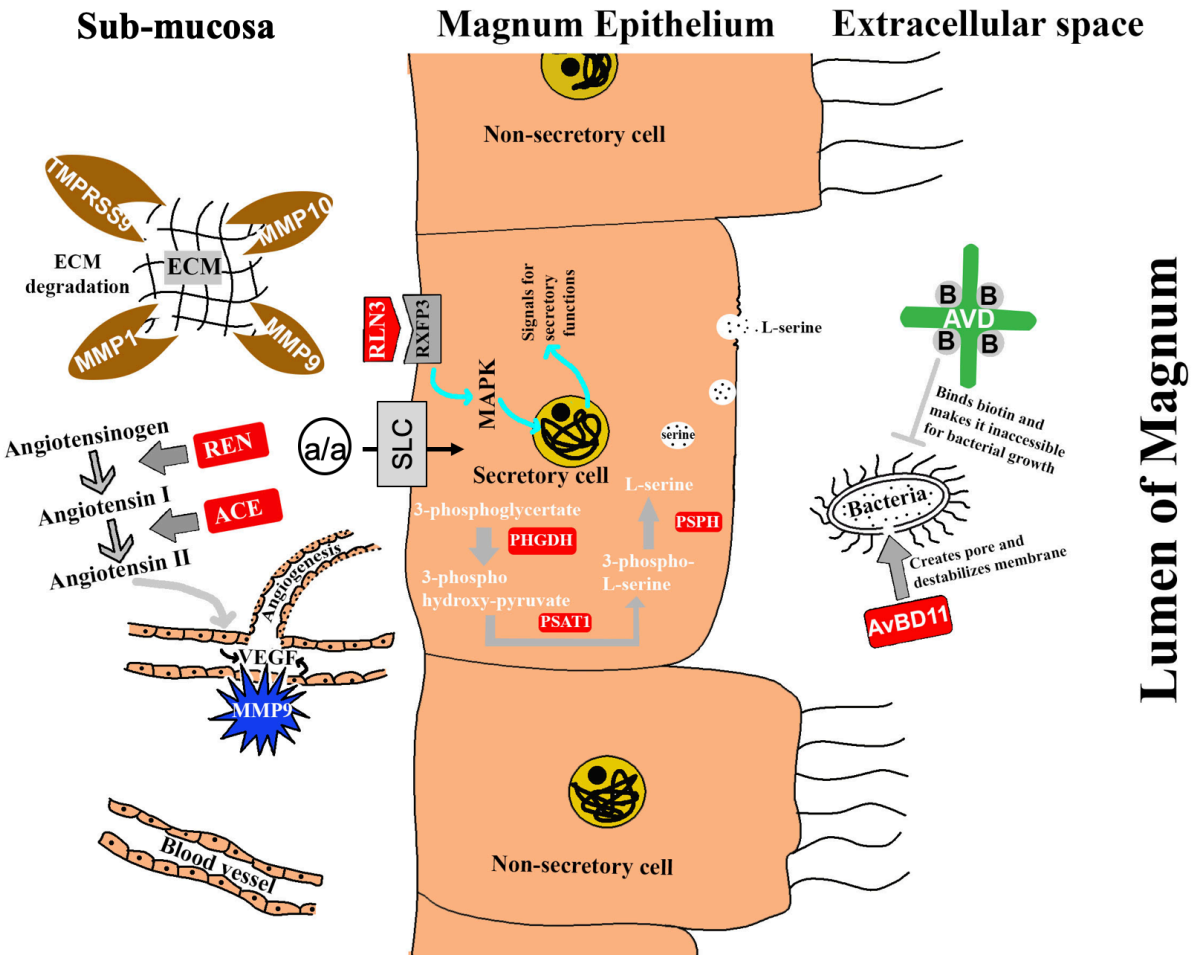

Figure 17 The proposed model on the functionality of the genes and biological pathways for the synthesis and secretion of the egg-white (albumen) proteins. TMPRSS9, MMP1, MMP9, MMP10 degrades the ECM. REN, ACE and MMP9 stimulate angiogenesis. SLCs transport the amino-acids across the epithelium for protein biosynthesis. PHGDH, PSAT1, and PSPH catalyzes the synthesis of L-serine. RLN3 triggers the secretion signaling. AVD and AvBD11 have antimicrobial activity.

Based on the functional analysis of the novel uterine genes identified in our study as well as previously reported (Jonchere et al., 2012; Brionne et al., 2014), we proposed a hypothetical model showing the identified genes and the cognate biological pathways involved in calcium transport for eggshell mineralization in the uterus (Figure 18). The massive amounts of Ca^{2+} ions required for eggshell mineralization are not reserved in the uterus but continuously supplied through the bloodstream (Brionne et al., 2014). Under the influence of estrogen, calcium stored in the medullary bones are mobilized into the bloodstream (Whitehead, 2004). From the blood

capillaries, Ca^{2+} ions move into the extracellular space and are transported in the uterine epithelium by passive transport towards lower concentration gradient maintained by calbindin 1 (Jonchere et al., 2012). Several molecules such as ATP2B1 and ATP2B2 directly transport Ca-ions; ATP1A1, ATP1B1, NKAIN4 participate in Na^{+}/K^{+} exchange across the cell; and KCNH1 regulates the efflux of K^{+} ions across the uterine epithelium (Brionne et al., 2014). Besides those reported genes, our findings suggest that Ca^{2+} ions are also transported actively across the uterine epithelium by some novel transporters such as ATP2C2, CALCB, and OTOP. The free Ca^{2+} ions influx from the circulation into the uterine epithelial cells further induces the release of Ca^{2+} ions from the intracellular calcium reserves (endoplasmic reticulum and Golgi apparatus) (Lemmens et al., 2001). This enormous amounts of intracellular Ca^{2+} ions are then carried by sodium-calcium exchangers (NCX), and plasma membrane Ca^{2+} ATPase (PMCA) into the extracellular matrix where they are combined with bicarbonate ions to initiate mineralization of the eggshell in the uterine lumen (Zhang et al., 2015).

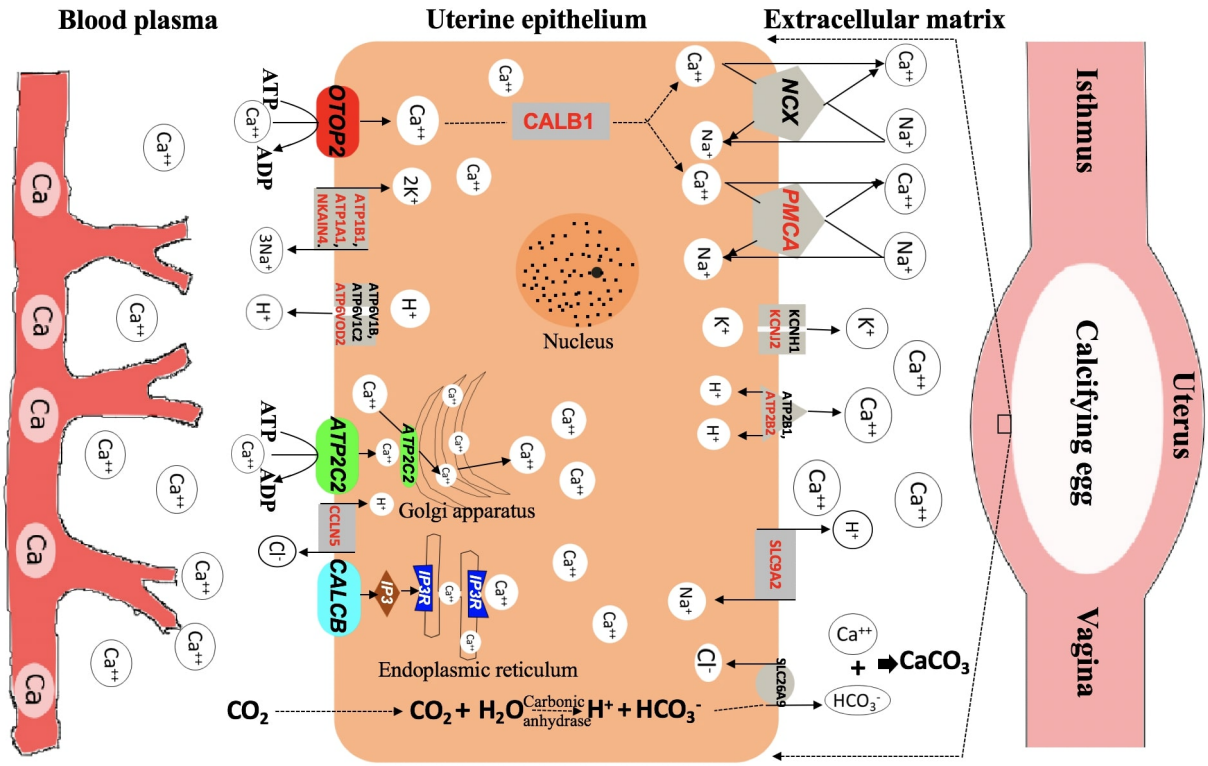

Figure 18 The proposed hypothetical model showing the identified genes and the cognate biological pathways involved in calcium transport for eggshell biomineralization in the uterus. The molecules with colored shapes and red-texts are novel genes detected in this study, whereas, those with grey texts and/or shape are genes described by previous studies (Jonchere et al., 2012; Brionne et al., 2104). From the blood capillaries, Ca^{2+} ions move into the extracellular space and are transported actively across the shell gland epithelium through some ion-transporters such as ATP2C2, CALCB, and OTOP. The calcium influx in the shell gland epithelium further induces the release of Ca^{2+} ions from intracellular calcium reserves such as endoplasmic reticulum and Golgi apparatus. This huge amount of intracellular Ca^{2+} ions from the shell gland epithelium are transported across by sodium-calcium exchangers (NCX) and plasma membrane Ca^{2+} ATPase (PMCA) into the extracellular matrix where they are combined with bicarbonate ions to initiate mineralization of the eggshell.

The novel molecules discerned by this study furthers our knowledge and understanding of the regulatory processes of egg formation in the oviduct. The expression profiles of the novel genes need to be examined in other breeds of laying hens that have differences in egg qualities (egg size, shell thickness, shell breaking strength, and shell shape). Such researches can confirm deeper links of the novel genes with egg formation and its qualities. Therefore, those novel genes can potentially be used as molecular markers for the egg quality traits. Breeders may perform selection based on quantitative traits with the updated knowledge of specific genes/proteins regulating the egg structure and quality. This knowledge of molecular markers can also be utilized by nutritionists to modulate the egg qualities through nutritional interventions.

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3. Sah N, Kuehu DL, Khadka VS, Deng Y, Jha R, Mishra B. RNA sequencing-based analysis of magnum specific genes regulating eggshell formation in laying hens (Unpublished).
4. Sah N, Kuehu DL, Wasti S, Khadka VS, Jha R, Mishra B. Role of Solute Carriers in the chicken egg formation (Unpublished).